INTERLEUKIN 1 AND FACTORS THAT AFFECT ITS ACTIVITIES *IN VIVO*

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ABSTRACT

Interleukin 1 alpha and beta are well-characterised cytokines that are thought to function in localised inflammation and immunoregulation. *In vitro* and *in vivo* studies on the biological activities of IL-1 also indicate a role in the tissue destruction of many inflammatory disease processes.

The detection of IL-1 in biological fluids is complicated by the presence of inhibitory and cytotoxic factors that interfere in bioassays. We have used immunoassays to measure IL-1 alpha and beta levels in human plasma. Detection of plasma IL-1 beta required a chloroform extraction step to remove factors that mask immunoreactive epitopes.

We have used these procedures to compare the levels of circulating IL-1 activity in normal individuals and patients with rheumatoid arthritis (RA). RA patients had raised plasma IL-1 beta concentrations that correlated with measures of disease activity. Individuals tested longitudinally also showed correlations between disease activity and plasma IL-1 beta. Interleukin 1 alpha levels however were not raised in RA when compared to age-matched healthy controls, but serial measurements in individual patients did correlate with some conventional measures of disease activity.

Biochemical studies showed that both IL-1 forms were bound to high molecular weight carriers in chloroform extracted plasma. The binding did not appear to interfere with bioactivity, but the complexes were identified by immuno- and bioassay only following chloroform extraction, suggesting the presence of other masking factors in plasma.

Using iodinated IL-1 a specific 43 kDa IL-1 beta binding protein was identified in plasma and synovial fluid. This protein was partially purified and characterised.

The primary translation product of IL-1 beta is a 31 kDa propeptide that requires processing to a 17 kDa peptide to become biologically active. In other studies IL-1 beta propeptide processing by monocytic cell membrane preparations was demonstrated. This proteolytic event could be inhibited by some protease inhibitors and a synthetic peptide designed from the amino acid sequence of the cleavage region of IL-1 beta propeptide.

CONTENTS

DECLARATION

I hereby declare that the data presented is the result of my own investigations, in the case of collaborative studies other contributing workers have been named. The thesis itself has been composed by the author.

Julie A Eastgate.

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ORIGINAL ARTICLES

At the time of submission three articles have been published based on the work described in this thesis:

Eastgate J A, Symons J A, Wood N C, Grinlinton F M, di Giovine F S, Duff G W. (1988) "Correlation of plasma interleukin 1 levels with disease activity in rheumatoid arthritis." Lancet, 2: 706-709.

Eastgate J A, Symons J A, Duff G W. (1990) "Identification of an interleukin-1 beta binding protein in human plasma." FEBS Lett, 260: 213-216.

Symons J A, Eastgate J A, Duff G W. (1990) "A soluble binding protein specific for interleukin 1 beta is produced by activated mononuclear cells." Cytokine, 2: 190-198.

Another has been accepted for publication;

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Reprints of these articles are enclosed inside the back cover.

ABBREVIATIONS

cDNA	complementary deoxyribonucleic acid
cm^2	centimetres square
CO_2	carbon dioxide
dCTP	deoxycytosine triphosphate
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
fmol	femtomoles
g	grams
kb	kilobase
kDa	kilodalton
l	litre
HPLC	high performance liquid chromatography
M	molar
mA	milliamps
ml	millilitres
mm	millimetres
mM	millimolar
mRNA	messenger ribonucleic acid
N	normal
ng	nanograms
nm	nanometres
NMR	nuclear magnetic resonance
pg	picograms
psi	pounds per square inch
RNA	ribonucleic acid
RNAse	ribonuclease
U	units
ug	micrograms
ul	microlitres
uM	micromolar
v/v	volume to volume
xg	times gravity
°C	degrees Celsius
<	less than
>	greater than
~	approximately

1. INTRODUCTION

1.1.a. GENERAL

Efficient activation of the immune system in response to an infective agent is essential for host defence. This activation process involves an adaptive response in which specific antigenic epitopes trigger the clonal expansion of T and B lymphocytes, and local mobilisation of phagocytic cells capable of removing foreign material in an innate response.

Adaptive immunity is initiated by uptake and presentation of antigen by accessory cells to T lymphocytes, and by the binding of antigen to surface immunoglobulin on B cells. T lymphocytes recognise processed antigen, associated with the cell surface major histocompatibility complex (MHC), via the T cell receptor (Springer, 1990). Two structural forms of MHC molecule exist. Class I MHC binds peptides synthesised endogenously such as viral components in infected cells, class II MHC processes exogenous antigen following uptake by endocytosis. The interaction between antigen presenting cells and T cells is strengthened by the involvement of surface adhesion molecules. The MHC/antigen complex is in turn recognised by two types of T cell: able to kill infected cells (cytotoxic T cells); or promote the expansion and differentiation of other activated cell types (helper T cells), in particular B cells. Class I MHC binds T cells via an association with the CD8 molecule, whereas class II interacts with CD4. As a result of specific activation and T cell help particular B cells undergo clonal expansion and antibody synthesis.

Activation of the cells recognising an antigen results in the selective expansion of a small number of lymphocyte clones. The production of an effective response is dependent on various cellular interactions, involving direct cell-cell contact and release of soluble factors such as cytokines. Cytokines are a group of low molecular weight peptide regulatory molecules (Green, 1989). Responsiveness of lymphocytes to cytokines results in proliferation, maturation and activation of primed cells.

The innate response is also highly dependent on soluble factors. Cytokines are involved in the recruitment of phagocytic cells, increasing local blood supply and vascular permeability, activating infiltrating cells

at the site of injury, and in remodelling damaged tissue once the infective agent has been eliminated.

Cytokines are important mediators of local cell activation, such as in regional lymph nodes during initiation of immune responses or at sites of tissue damage. However they also have systemic roles and can act in a hormone-like manner on entering the blood or lymphoid circulation. This is seen particularly in the acute phase response where infection results in a number of metabolic changes, including fever and acute phase protein production in liver cells.

Cytokines have long been regarded as modulators of the immune response but increasing evidence suggests that they may also play an important role in normal homeostatic functions, connective tissue remodelling and haematopoiesis. Their ability to influence cellular proliferation and differentiation indicates a possible function in normal growth and development.

1.1.b. Cytokines and interleukins

Cytokines are inducible peptides with wide ranging effects on many cell types, acting through specific receptor molecules. They may affect proliferation, differentiation and function depending on the cell type and the particular *in vivo* environment. Examples of cytokine families include interleukins, tumour necrosis factors, colony stimulating factors, interferons and various other peptide growth factors.

The interleukins are a group of cytokines originally described as mediating communication between leukocytes. Increased understanding of their biological functions has led to interleukins being re-defined as: being inducible in leucocytes and other cell types, involved in the inflammatory response, and having a known amino acid sequence.

1.2. INTERLEUKIN-1 (IL-1)

Interleukin 1 is a well characterised cytokine thought to be a central mediator in the mobilisation of the immune response and localised

inflammatory reactions, it is also implicated as having a role in various normal metabolic processes. Though initially described as a product of activated macrophages, there are many cellular sources that respond to different stimuli (Table 1.2.1.). Interleukin 1 exerts wide variety of effects, through activation of specific receptor molecules, and has autocrine, paracrine and endocrine functions.

Recently many advances have been made in understanding the molecular and cell biology of IL-1 and the role of inhibitory factors in regulating its effect.

1.2.a. History

Interleukin 1 was initially described as a protein able to produce a febrile response when injected into animals (Atkins, 1955) and was named endogenous pyrogen. Following this, various macrophage products were identified and named according to their biological activity. In 1972 murine lymphocyte activating factor (LAF) was shown to act as a co-mitogen for thymocytes (Gery and Waksman, 1972). A B cell activating factor (BAF) was identified in 1979 (Wood and Cameron, 1978) and leukocytic endogenous mediator (LEM, Kampschmidt *et al*, 1973) was well characterised as an inducer of acute phase changes. Other activities described include osteoclast activating factor (Dewhirst *et al*, 1985), mononuclear cell factor (MCF; Krane *et al*, 1982) that stimulated prostaglandin and collagen synthesis by fibroblasts, and pig catabolin (Saklatvala and Dingle, 1980) capable of inducing cartilage breakdown *in vitro*.

The term interleukin 1 was introduced in 1979, since then the cloning of IL-1 has established the common identity of these factors and has verified the activities of this cytokine.

1.2.b. Cellular sources and inducers of IL-1 production

Many cell types have been shown to produce IL-1, however the traditional laboratory source is from peripheral blood monocytes. A wide range of specialised cell types such as T and B lymphocytes, smooth muscle,

endothelial and various brain cell types have been shown to synthesise IL-1. Cellular sources of IL-1 are summarised in Table 1.2.1.

Resting monocytes produce no IL-1, however, stimulation by a number of agents will rapidly induce IL-1 mRNA and protein synthesis. Bacterial derivatives, such as cell wall components, are direct activators of IL-1 production, as are viral and yeast particles. Non-infective agents for example urate, silica and asbestos can induce IL-1. Products of the immune system itself such as antigen-antibody complexes other cytokines and complement components also act as stimuli. In *in vitro* laboratory systems stimuli such as lipopolysaccharide (LPS), phorbol myristic acetate (PMA), calcium ionophores and ultra violet (UV) irradiation are commonly used with different cell types (di Giovine and Duff, 1990).

Interleukin 1 can also be induced by itself and by other cytokines such as tumour necrosis factor (TNF) alpha, interferons (IFNs) alpha and beta and colony stimulating factors (CSFs). Lymphocytes activated by exposure to antigen can promote IL-1 synthesis by production of other cytokines or by direct cell-cell contact primarily between activated T cells and macrophages.

1.2.c. Biological effects of interleukin 1

The effects of IL-1 *in vitro* and *in vivo* were confirmed when the recombinant proteins became available. The observed properties are consistent with IL-1 having a role in both local defence, at a site of infection or injury, and in generalised activation of an immune and systemic non-immune response. The biological effects of IL-1 are summarised in Table 1.2.2., the molecule is shown to have an extensive range of actions involving the maturation, growth and differentiation of many cell types.

Interleukin 1 also acts at a local level in the lymph node to activate cells involved in the immune response, such as T cells via the induction of IL-2, its receptor and IL-4. In the human system antigen, is thought to activate both T cells and accessory cells resulting in IL-1 production and therefore clonal expansion of the activated lymphocytes (Mizel, 1987). Interleukin 1 is also thought to act as a helper factor during B cell

Table 1.2.1. CELLULAR SOURCES OF IL-1

Monocytes	Blood Placental
Tissue macrophages	Alveolar Kupffer cells Synovial cells Peritoneal macrophages
Lymphocytes	T helper cells B cells Natural killer (NK)/ Large granular lymphocytes (LGL)
Vascular cells	Smooth muscle cells Endothelial cells
Brain cells	Astrocytes Microglia Glioma cells
Skin cells	Keratinocytes Langerhans cells
Other cell types	Dendritic cells Kidney mesangial cells Neutrophils Fibroblasts Chondrocytes Corneal epithelium Thymic epithelium Noradrenergic neural cells

Based on di Giovine *et al*, 1988

Table 1.2.2. THE EFFECTS OF IL-1

CELL TYPE	*IN VITRO*	*IN VIVO*
Immune System		
T cells	Cytokine induction (IL-2, IL-4) IL-2 receptor expression Radioprotection	
B cells	Proliferative response Augment antibody production Chemotactic	
Monocytes	Prostaglandin production IL-1 induction Cytotoxic Chemotactic	
N K cells	Synergise with IL-2 in cytotoxic activity	
Basophils	Degranulation and histamine release	
Neutrophils	Chemotactic Adhesion to endothelial cells Degranulation, thromboxane synthesis, reactive oxygen production	Release from bone marrow
Central Nervous System		
Astrocytes	Proliferation	
Hypothalamic cells	Prostaglandin production	Fever induction
Liver		
Hepatocytes	Decreased albumin synthesis Increased synthesis of some acute phase proteins	Acute phase protein production Altered plasma metal levels Decreased cytochrome P_{450} levels
Musculo skeletal system		
Bone	Resorption Osteoclast activation Osteoblast mitogenesis	Bone marrow radioprotection Cytokine production in bone marrow

Table 1.2.2. continued

CELL TYPES	*IN VITRO*	*IN VIVO*
Cartilage	Resorption Decreased proteoglycan and collagen synthesis Proteoglycan release	Articular proteoglycan degradation
Fibroblasts	Proliferation Prostaglandin and collagenase production CSF induction	
Synoviocytes	Prostaglandin and collagenase production	
Muscle	Proteolysis	Proteolysis
<u>Vascular tissue</u>		
Endothelial cells	Proliferation Prostaglandin production Procoagulant activity Increased adhesiveness CSF induction	
Vascular smooth muscle	IL-1 production in response to IL-1	
<u>Other effects</u>		
Cytotoxic/ cytostatic	To pancreatic beta islet and some transformed cells	Increased insulin production
	Eosinophil degranulation Keratinocyte proliferation	Induces slow wave sleep Causes hypotension Increases steroid levels Increases survival in immune suppressed mice Suppresses appetite Increased sodium excretion Increased cardiac output Hypercalcaemia

Taken from di Giovine *et al*, 1988; Dinarello 1989; Durum *et al*, 1985

activation (Durum *et al*, 1985).

At a systemic level IL-1 is involved in the acute phase response. For many years the ability of IL-1 to induce fever has been known, and also to stimulate hepatocyte production of acute phase reactants including metalloproteins, and alter serum zinc and iron levels. Experimental animal models in which IL-1 has been administered intravenously result in effects comparable to those seen in the human acute phase response (reviewed by Dinarello, 1989).

1.3. THE INTERLEUKIN 1 MOLECULE

Interleukin 1 is produced as two distinct gene products, alpha and beta, that have similar activity profiles and receptor binding characteristics, these can be differentiated biochemically on the basis of isoelectric focusing point (pI). The genes encode propeptides that are subsequently cleaved to give the mature protein. Only processed IL-1 beta is biologically active while both the IL-1 alpha propeptide and cleaved C-terminus (17 kDa) have receptor affinity and biological activity.

1.3.a. The interleukin 1 gene

In 1984 the nucleotide sequence for murine IL-1 alpha cDNA was reported by Lomedico *et al*, shortly followed by that for human monocytic IL-1 beta (Auron *et al*, 1984). March *et al* (1985) then isolated and expressed two human IL-1 cDNAs. The IL-1 alpha cDNA encoded a 271 amino acid (30.6 kDa) propeptide which was proteolytically cleaved to give a 159 amino acid (17.5 kDa) mature acidic (pI 5) molecule. The IL-1 beta cDNA codes for a 269 amino acid (30.75 kDa) precursor that is processed to the 153 amino acid (17.4 kDa) neutral and biologically active form. The human IL-1 cDNAs have been expressed in *Escherichia coli* (*E coli*), simian COS cells and *Xenopus laevis* oocytes (Gubler *et al*, 1986; Rosenwasser *et al*, 1986; Auron *et al*, 1984). Comparison between the human cDNAs shows 45% homology at the nucleotide level suggesting that the two forms arose by a gene duplication event (March *et al*, 1985).

Interleukin 1 alpha and beta have now been characterised at the gene level, and both are known to be located on chromosome 2 (Lafarge *et al*, 1989; Webb *et al*, 1986). The IL-1 genes have a similar overall structure with seven exons and six introns. The human IL-1 alpha gene, sequenced by Furutani *et al* (1986), is 10.2 kb long and has similar structural organisation to the murine IL-1 alpha gene (Telford *et al*, 1986). The human IL-1 beta gene, (Bensi *et al*, 1987) spans a 7.5 kb region. Analysis of the two human genes shows that both have comparable exon length and splice site locations. The N terminal cleaved part of the propeptides are encoded by exons 2, 3 and 4, with the mature protein within exons 5, 6 and 7. It has been postulated that the IL-1 beta gene arose from retrotransposition of the alpha gene by Clark *et al*, (1986), who identify a slightly larger gene form of 9.7 kb.

1.3.b. Gene expression

In unstimulated cells IL-1 protein can not be found indicating that neither gene is constitutively expressed. However activation by a suitable stimulus results in the rapid appearance of mRNA for both IL-1 alpha and beta. In activated mononuclear cells IL-1 beta mRNA is approximately ten times more abundant than that for IL-1 alpha (March *et al*, 1985). However when total production of the respective proteins is considered IL-1 alpha is the predominant form (Lonnemann *et al*, 1990; di Giovine *et al*, submitted). It has therefore been suggested that IL-1 alpha mRNA may undergo more efficient translation (Dinarello, 1989).

Analysis of the IL-1 genomic sequence identifies potential regulatory regions, the conservation of intron structure between the two forms suggests that these may be involved in gene control (Bensi *et al*, 1987). Searches for promoter motifs has shown CAAT and TATA box sequences in the IL-1 beta gene. The IL-1 alpha gene, however, has a poor TATA box and no obvious CAAT (Clark *et al*, 1986). Other regulatory motifs have been shown in the IL-1 genes including glucocorticoid regulatory elements, regions containing OCT 1 sites, and binding sites for SP-1 like factors.

Studies on regulation of gene expression showed transient expression of

IL-1 beta in response to LPS, thought to be mediated by both transcriptional and post-transcriptional mechanisms (Fenton *et al*, 1987). Alterations in the stability of IL-1 mRNA is thought to be involved in the regulatory mechanisms (Fenton *et al*, 1988). A transcriptional activator has been identified that is able to bind to upstream sequences of the pro IL-1 beta gene (Clark *et al*, 1988). Arend *et al* (1989a) have suggested that a transcriptional repressor of IL-1 beta expression is produced by *in vitro* aged monocytic cells, and that production of this factor may be linked to their differentiation to macrophages. A recent report (Schindler *et al*, 1990) demonstrates transcription of the IL-1 beta gene without the induction of protein synthesis in blood mononuclear cells. Work by Turner *et al* (1988) has shown that activation of the myelomonocytic cell line, THP-1 results in a similar increase in transcription rate for IL-1 alpha and beta genes. They demonstrate that the higher steady state level of IL-1 beta mRNA in THP-1 cells and monocytes is as a result of post-transcriptional control mechanisms extending the half life of the mRNA (Turner *et al*, 1989). A similar effect is seen in response to interferon gamma treatment (Burchett *et al* 1988). Prostaglandins are reported to reduce the expression of IL-1 by a post-transcriptional mechanism (Knudsen *et al*, 1986).

In vivo the systemic IL-1 level is potentially regulated by a complex feedback network involving neuroendocrine hormone effects. Interleukin 1 is able to stimulate adrenocorticotrophic hormone production (ACTH), either by a direct action on the anterior pituitary or by stimulating the release of corticotrophin releasing factor from the hypothalamus (reviewed in Lumpkin, 1987). Expression of the gene encoding pro-opiomelanocortin, the precursor of both ACTH and the neuropeptide alpha-melanocyte stimulating hormone (alpha MSH) is enhanced by IL-1 (Brown *et al*, 1987). ACTH acts on the adrenal glands to promote corticosteroid production which, in turn, can inhibit IL-1 synthesis (Besedovsky *et al*, 1986; Knudsen *et al*, 1987). Alpha MSH is able to inhibit IL-1 effects both *in vitro* and *in vivo* (Cannon *et al*, 1986; Robertson *et al*, 1988).

1.3.c. Interleukin 1 protein structure

Comparison of the sequences of the IL-1 protein molecules shows 62% homology between human and murine IL-1 alpha but only 26% between human alpha and beta forms (March *et al*, 1985). Of the identical residues the majority are towards the C terminal of the 17 kDa molecule, though some homology is also seen in the first 80 amino acids of the precursor peptide.

The structure of the interleukin 1 proteins has been analysed by various groups. The initial crystal structure determination of mature IL-1 beta was reported by Priestle *et al* in 1988, the molecule is composed of 12 beta strands interacting via a complex network of hydrogen bonds to form a barrel like structure. The overall shape was described as a distorted tetrahedron, each face being triangular with hydrophobic side chains filling the interior. The same group in 1989 described a refinement of the model from studies at higher resolution (Priestle *et al*, 1989), they confirmed the original structure and also described a high degree of conservation of amino acids located at the interior. There was little clustering of invariant surface amino acids and so definition of the area involved in receptor recognition was not possible. Finzel *et al* (1989) showed a similar structural model with predominant beta strands and no alpha helix. To account for the high affinity of IL-1 for its receptor it is proposed that a region of large surface area, containing several peptide segments, is necessary for the interaction. The IL-1 alpha crystal structure has also been reported (Graves *et al*, 1990) and shows a capped barrel shaped molecule with similar topology to IL-1 beta. The N-terminal regions however are positioned differently, possibly explaining why the additional propeptide residues in the IL-1 beta precursor interfere with receptor binding.

Interleukin 1 propeptide structure has only been examined for the beta form, using circular dichroism spectra (Hazuda *et al*, 1989). The data suggest that mature and precursor forms both have similar secondary structure, the latter though having some alpha helical regions. The IL-1 beta propeptide may have a more open tertiary structure which would account for its increased protease susceptibility in comparison to the mature form.

Considerable effort has gone into defining functional domains within the IL-1 molecules. By introducing point mutations various reports have shown that isolated amino acid changes may result in alterations in local protein structure, but in most cases overall folding and biological activity remain unchanged (Driscoll *et al*, 1990; Gronenborn *et al*, 1986). The histidine at position 30 (MacDonald *et al*, 1986) of IL-1 beta was shown to be important in stabilising part of the protein structure. A mutation of Arg^{127} to a glycine was demonstrated significantly to reduce IL-1 beta bioactivity while resulting in a smaller disruption of receptor binding ability (Gehrke *et al*, 1990). Muteins with enhanced activity have been produced (Huang *et al*, 1987), and it is suggested that this effect may be due to altered stability, or differences in interactions with the receptor molecule. It has been proposed that other mutations, that reduce IL-1 bioactivity, are likely to act by disrupting the tertiary structure (Priestle *et al*, 1989).

Truncated IL-1 molecules have been tested for biological activity and in the majority of cases have shown that loss of a few amino (N) or carboxyl (C) terminal amino acids considerably reduces the function of both forms (DeChiara *et al*, 1986; Mosley *et al*, 1987a; Zurawski *et al*, 1986). This may be due to disruption of the barrel-like structure of the folded molecule (Finzel *et al*, 1989), particularly in the case of the C terminal region where antibody binding has been shown not to affect interaction with the receptor (Lillquist *et al*, 1988). Other antibody studies (Massone *et al*, 1988) show that binding to regions 133-148 and 251-269 inhibited IL-1 beta activity in the thymocyte proliferation assay and prevented fibroblast secretion of PGE_2.

A synthetic peptide corresponding to amino acids 163-171 of IL-1 beta was shown to have immunostimulatory but not inflammatory activity (Antoni *et al*, 1986), however the peptide was only active at very high concentrations. A tripeptide analogue of IL-1 beta (amino acids 193-195) was found to antagonise the effect of the parent molecule in a rat paw hyperalgesic model (Ferreira *et al*, 1988), but did not show agonist or antagonist IL-1 effect in *in vitro* assays. The best characterised synthetic peptide studied (Palaszynski, 1987) was composed of residues 237-269 of IL-1 beta. The

peptide antagonised IL-1 in the thymocyte assay and bound to the receptor.

1.4. THE CELL BIOLOGY OF INTERLEUKIN 1

1.4.a. Cell localisation

Various groups have studied the subcellular localisation of IL-1 using a number of techniques. By fractionating cells to produce crude organelle preparations, Matsushima *et al* (1986c) showed that in monocytes 95% of the IL-1 could be found in the cytosol. The remaining activity was shown in membrane and particulate fractions following detergent extraction. A similar study by Bakouche *et al*, (1987a) identified IL-1 with crude plasma membranes and with lysosomal and cytosolic compartments. Part of this activity appeared to be in the inactive precursor form. Interleukin 1 could not be localised to the endoplasmic reticulum. Conlon *et al* (1987) showed cell surface labelling for IL-1 alpha but not IL-1 beta by flow cytometry.

Immunofluorescent staining of the stimulated U937 line co-localised IL-1 beta with cell microtubules (Baldari and Telford, 1989). By immunoelectron microscopy IL-1 beta was predominantly found in the cytoplasmic ground substance, no staining was identified at the plasma membrane or within the endoplasmic reticulum, Golgi apparatus or secretory vesicles (Singer *et al*, 1988).

Various groups have identified IL-1 bioactivity at the plasma membrane, though neither IL-1 molecule has an obvious hydrophobic spanning region (Kurt-Jones *et al*, 1985; Beuscher and Colten *et al*, 1988). The activity was inhibitable only with antibodies against IL-1 alpha (Fuhlbrigge *et al*, 1988b). However, this may be due to leakage of IL-1 precursor molecules from the cell following insufficient parafomaldehyde fixation (Minnich-Carruth *et al*, 1989; Suttles *et al*, 1990a). Fuhlbrigge *et al* (1988a) demonstrated, by cell labelling, a transient association of IL-1 alpha with cell membranes in transfected fibroblasts. Another group, also using transfected fibroblasts, found no membrane IL-1 using a fractionation protocol designed to wash off loosely bound proteins (Young *et al*, 1988). Interleukin 1 alpha propeptide is also suggested to associate with the plasma membrane via a lectin-like

interaction with integral proteins (Brody and Durum, 1989).

1.4.b. Interleukin 1 processing

Interleukin 1 alpha and beta are both produced as large molecular weight (31 kDa) precursors that are cleaved at a specific site to give mature C-terminal molecules. Incorrect processing that either leaves or deletes extra amino acids has been shown, in the case of IL-1 beta, to affect the bioactivity of the resultant product (Black *et al*, 1988). The majority of work concerning the mechanisms involved in IL-1 cleavage has concentrated on the beta form. The IL-1 beta primary translation product has no receptor binding or biological activity, the IL-1 alpha propeptide however elicits similar responses to the mature molecule (March *et al*, 1985; Hazuda *et al*, 1989).

Both IL-1 gene products are 31 kDa precursor molecules. Interleukin 1 alpha is cleaved from a 271 amino acid (aa) propeptide to the 159 amino acid mature form at the bond between arginine ^{112} and serine ^{113} (Gubler *et al*, 1986). Pro IL-1 beta has 269 amino acids and is processed to the 153 aa mature form at the aspartate ^{116}-alanine ^{117} site (Cameron *et al*, 1985). The propeptide molecules have a high level of homology in the N terminal region up to amino acid 80, however this area has no obvious signal sequence or membrane spanning domain though IL-1 is secreted *in vitro*. Near the proteolytic site a short sequence of polybasic residues are found that have been compared to known processing sites on other prohormones (March *et al*, 1985). The bond cleaved in the IL-1 beta precursor is an uncommon substrate for many proteolytic enzymes, being preceded by valine, histidine and aspartate (Black *et al*, 1988).

The interleukin 1 processing event is highly inefficient, intracellular IL-1 bioactivity has long been known to exceed extracellular activity following LPS stimulation (Gery and Lepe Zuniga, 1984). Studies concerning the kinetics of IL-1 protein production and release have shown that IL-1 alpha is predominantly cell associated, 95% being retained within the cell following 18 hours of culture. Interleukin 1 beta, however, accumulates

within the cell during the first two hours of culture and is then released resulting in high extracellular levels (di Giovine *et al*, submitted). Considerable cellular accumulation of IL-1 beta immunoreactivity is seen even if secretion occurs, much of this may be the inactive 31 kDa form. Auron *et al* (1987) estimate a ratio of 5:1 for intracellular to extracellular IL-1, and suggest that this is due to the absence of an efficient signal sequence to facilitate transport. Studies measuring IL-1 production and secretion by immunoblotting show that mature IL-1 beta (17 kDa) is only found in culture supernatants. However at the same time much larger amounts of extracellular and intracellular propeptide have been reported (Bomford *et al*, 1987). It is difficult to determine whether the presence of extracellular propeptide is the product of specific secretion or a result of cell death.

Certain stimuli are able not only to induce IL-1 production but also to increase the amount of secreted bioactive protein. Agents showing this effect such as silica (Lepe-Zuniga and Gery, 1984) and monosodium urate (di Giovine *et al*, submitted) are often particulate in nature and may cause membrane damage, resulting in leakage of IL-1 protein from cells. The production of large amounts of IL-1, usually for purification purposes, has identified a superinduction protocol using cycloheximide (Mizel, 1982). Pulse labelling of superinduced cells showed that a large amount of the radiolabelled IL-1 could be detected extracellularly and the majority was in a low molecular weight form (Giri *et al*, 1985). This phenomena has been suggested to result from a synergistic effect of the inducing agents leading to high IL-1 levels, and a cytotoxic effect resulting in release of greater levels of extracellular mature protein.

The presence of processed 31 kDa IL-1 in culture supernatants has led to the suggestion that an enzyme responsible for cleavage is located either at the cell surface or is secreted by the cell (Black *et al*, 1988; Bomford *et al*, 1987). Hazuda *et al* (1988) however could show no processing of added precursor by mononuclear cells in culture. Reports of specific processing of pro IL-1 beta by cell membrane preparations (Black *et al*, 1988) and cytosolic fractions (Kostura *et al*, 1989; Black *et al*, 1989b) have appeared.

The gene encoding the cleavage enzyme may show tissue specific expression. Both IL-1 alpha and IL-1 beta genes have also been transfected into fibroblasts and no observable processed or secreted product was found (Fuhlbrigge *et al*, 1988a; Young *et al*, 1988). In keratinocytes pro IL-1 beta is also synthesised but is not converted to the mature molecule (Kupper *et al*, 1986). The enzymes demonstrated in cytosol and membrane preparations have not been found in non-myeloid cell types (Kostura *et al* 1989; Black *et al*, 1988).

It has been postulated that in certain situations, non-specific proteases may be involved in IL-1 processing, resulting in production of intermediates with extra N terminal amino acids (Hazuda *et al*, 1990). Such proteolytic activity is described in inflammatory joint exudates from arthritic patients and in lavage fluids from individuals with sarcoidosis.

1.4.c. Secretion of interleukin 1

Little is known about the mechanisms involved in the secretion of IL-1 from cells, as neither IL-1 molecule has a recognisable hydrophobic signal sequence (March *et al*, 1985). It is unclear whether proteolytic processing occurs before or after secretion, and the relevance of membrane damage as a means of release in the *in vivo* situation is unknown.

Some reports describe a dissociation between the production of intracellular IL-1 and its secretion, the data is summarised by Cavaillon and Haeffner-Cavaillon (in press). The majority of studies use stimuli such as the lipid A component of LPS or activation by cellular adhesion molecules to dissociate synthesis and secretion events (Cavaillon *et al*, 1989; Cavaillon *et al*, 1990). Many of these reports use bioactivity as an assessment of cell associated and secreted IL-1 levels, and so do not differentiate between IL-1 alpha and beta or identify the IL-1 beta propeptide. One report, however, describes the enhancement of IL-1 beta secretion by recombinant complement 5a (C5a) in which the cell associated IL-1 beta levels are not altered (Cavaillon *et al*, 1990). Bacle *et al* (1990) reported that monomeric complement stimulates IL-1 production but not release, whereas the polymeric

form induces both. A similar effect has also been demonstrated for interferon gamma augmentation of IL-1 beta secretion (Cavaillon and Haeffner-Cavallion, in press). This effect is seen with different types of stimuli, liposome encapsulated LPS also results in normal levels of intracellular IL-1 but no secreted molecule (Bakouche *et al*, 1987b). Kern *et al* (1988) have shown that dexamethasone is able dramatically to inhibit the secretion of IL-1 beta while only moderately reducing its synthesis.

Burchett *et al* (1988) showed that stimulation of *in vitro* aged monocytes resulted in lower levels of total IL-1 production, and in the proportion of IL-1 beta protein that was secreted when measured by bioassay or immunoblotting. Other studies showed that when activation of IL-1 production occurred in unstimulated cells it was characterised by a transient increase in intracellular activity with no apparent secretion (Mizel and Rosenstreich, 1979; Lepe-Zuniga and Gery, 1984).

Secretory proteins are commonly translocated across the membrane and into the lumen of the endoplasmic reticulum (ER), during or shortly after synthesis. Involved in this process are integral protein signal sequences and receptor-like components in the cytosol and on the target membrane (reviewed by Verner and Schatz, 1988).

A protein is directed to its target membrane by a signal sequence that is usually located at the N terminus (Gierasch, 1989). This sequence is likely to be recognised as a characteristic secondary or tertiary structure. The signal region binds to the cytosolic face of the ER, and the growing protein is translocated across the membrane as a loop that opens when the C terminus reaches the luminal side. Secretory proteins such as ovalbumin (Meek *et al*, 1982) are known in which an internal signal sequence is utilised.

A number of cytosolic components are necessary for the translocation event. A signal recognition particle binds to the emerging signal sequence during translation, this may result in arrest of further protein synthesis. The complex then associates with a docking protein on the ER, where the signal sequence can interact with a membrane signal sequence receptor (Walter

et al, 1984). It is thought that other cytosolic chaperon molecules are involved in translocation, these may be important in maintaining the loosely folded conformation of the nascent protein allowing it to pass through the membrane (Pelham, 1989; Flynn *et al*, 1989). The signal sequence is usually removed by integral membrane proteases on the luminal side of the ER.

Within the ER a protein is able to fold into its mature tertiary structure and oligosaccharides may be added. It is transported to the *cis*-face of the Golgi apparatus where oligosaccharides are modified. When complete the protein leaves the *trans*-face of the Golgi via the budding off of transport vesicles, these are directed either to secretory vesicles or to the plasma membrane (Schwartz, 1990).

Interleukin 1 molecules have no characteristic signal sequence, though Auron *et al* (1985) used hydropathy index data to suggest that a 17 residue N terminal hydrophobic region may substitute for this function. In comparison the TNF molecule has a long hydrophobic segment that is thought to promote efficient secretion. It has been suggested that IL-1 is therefore released by means of an alternative mechanism (Furutani *et al*, 1985).

Reticulocyte lysate preparations have been shown to be incapable of inserting the IL-1 propeptide into dog pancreas microsomes (Lomedico *et al*, 1984; Suttles *et al*, 1990b). Various studies indicate that IL-1 forms are not present in the ER, Golgi or secretory vesicles (Singer *et al*, 1988). Baldari *et al* (1987) report that fusion of a characteristic leader sequence onto IL-1 beta results in high secreted levels of the correctly processed molecule. However other workers were unable to effect secretion from *E. coli* when IL-1 beta was fused to an efficient signal region (Denefle *et al*, 1989).

A number of secretory pathways for IL-1 have been suggested. The debated presence of membrane IL-1 has resulted in the theory that a membrane linked intermediate may play a role in release of the alpha form (Bakouche *et al*, 1987a). In the case of IL-1 alpha the propeptide has been demonstrated to associate with the membrane via a lectin-like interaction (Brody and Durum, 1989). This membrane form is suggested to be involved in the secretory

pathway by which IL-1 alpha is released from the cell.

A report by Bursten *et al* (1988) showed that both IL-1 alpha and beta precursors may be covalently linked to myristic acid during synthesis, this modification was lost with removal of the pro-peptide portion. Myristylation is thought to occur co-translationally (Sefton and Buss, 1987) and to direct proteins to the plasma membrane (Pellman *et al*, 1985).

Another modification reported for IL-1 involves precursor phosphorylation, which may also provide some localisation signal. Weiel *et al* (1986) showed that LPS stimulation of macrophages resulted in increased phosphorylation of a number of proteins, the highest level being seen in a 33 kDa species. Two reports of IL-1 precursor phosphorylation have since been published, Beuscher *et al* (1988) identified residue Ser^{90} in IL-1 alpha as a ^{32}P labelled site and Kobayashi *et al* showed that the IL-1 alpha molecule was phosphorylated at ten fold higher levels than IL-1 beta. Labelling was not seen in mature IL-1. Beuscher *et al* report that phosphate labelled IL-1 alpha localises to the lysosomes, in agreement with Bakouche *et al* (1987a) who propose cleavage in and secretion from these organelles. The second report suggests that pro IL-1 alpha is more susceptible to proteolytic cleavage following phosphorylation which may serve as a signal for processing.

The observed association of pro IL-1 beta with tubulin has led to the suggestion that direct transport to the cell surface may be mediated by the microtubular network (Baldari and Telford, 1989). The role of tissue specific mechanisms has also been questioned, prompted by the co-localisation of IL-1 in keratinocytes with the secretory lamellar bodies (Didierjean *et al*, 1989).

1.4.d. Novel secretory pathways

A group of secretory proteins has recently been identified which lack the classical signal sequence and may follow a novel secretory pathway (Muesch *et al*, 1990). Members of this group include IL-1, acidic and basic fibroblast growth factors and platelet derived growth factor. These are present at high intracellular levels, indicative of inefficient secretion, and do not undergo modifications that usually occur in the ER or Golgi.

Evidence for a novel pathway is provided by Rubartelli *et al* (1990). They propose that IL-1 is selectively located in intracellular vesicles that may be part of the endocytic pathway. These fuse with the plasma membrane in a temperature and calcium dependent manner and the precursor molecule is cleaved on release from the cell. An involvement of calcium in IL-1 secretion has also been suggested by Suttles *et al* (1990b) who found that calcium ionophores enhanced release and processing.

1.5. THE INTERLEUKIN 1 RECEPTOR (IL-1 R)

Though IL-1 alpha and beta have little sequence homology both have comparable actions and appear to act through a common receptor. Initial work to identify the receptor led to the cloning of an 80 kDa protein with high affinity (dissociation constant 10^{-10}M) for both IL-1s. Since then further studies have shown the existance of other IL-1 binding molecules. The best characterised of these is a 60-70 kDa molecule that appears to act as a distinct receptor on B cells and monocytes.

1.5.a. The 80 kDa interleukin 1 receptor

The 80 kDa IL-1 receptor has been identified on a number of cell types (Dower and Urdal, 1987). Commonly, studies use human or murine T cell or fibroblast lines, but the receptor molecule has also been identified in tissues such as cultured synovium (Chin *et al*, 1988), human and murine neutrophils (Paganelli-Parker *et al*, 1989) and rat brain sections (Farrar *et al*, 1987).

The initial report identifying the 80 kDa IL-1 binding protein was published by Dower *et al* in 1985. They showed that radiolabelled IL-1 could be cross linked to a molecule on a murine T lymphoma line, and estimated that there were 500 receptors/cell with an affinity constant of approximately 10^{-10}M. Further work showed that this receptor bound both IL-1 alpha and beta (Dower *et al*, 1986). At the same time Bird and Saklatvala (1986) had identified a common class of receptor for IL-1 alpha and beta on connective tissue cells. The rate of association of ligand to receptor is very slow, and

once bound IL-1 does not dissociate (Kilian *et al*, 1986). The protein has been solubilised and its characteristics as an isolated molecule shown to be similar to the native form (Paganelli *et al*, 1987).

Analysis of the receptor showed that the protein was glycosylated, treatment with N-glycanase reduced its molecular weight to 62 kDa (Urdal *et al*, 1988). However an antiserum capable of immunoprecipitating the IL-1/IL-1R complex has identified a 52-56 kDa unprocessed form by *in vitro* translation that binds ligand (Rangnekar and Plate, 1988). The receptor binds the IL-1 alpha but not the beta propeptide (Mosley *et al*, 1987b).

1.5.b. Cloning and characterisation of the IL-1R

The murine IL-1 receptor was cloned by direct expression from mRNA isolated from the EL-4 line (Sims *et al*, 1988). The cDNA sequence identifies a protein of molecular weight 64.6 kDa, a similar size to that estimated for the deglycosylated receptor. The extracellular N-terminal segment is 319 amino acids long and has seven potential N-linked glycosylation sites. It forms three domains that are comparable to those seen in members of the immunoglobulin superfamily. The transmembrane region consists of a stretch of 21 uncharged amino acids, while the cytoplasmic domain has 217 residues and includes a potential protein kinase C phosphorylation site. Purification of the IL-1 receptor protein and amino acid sequencing of protease fragments confirmed the identity of the cloned molecule (Stern *et al*, 1989). Transfection of the murine IL-1R cDNA into Chinese hamster ovary cells resulted in expression of the molecule at the cell membrane and enhanced functional responses to IL-1 (Curtis *et al*, 1989). The human 80 kDa receptor was cloned by Sims *et al* (1989) and shown to have similar structural characteristics to the murine molecule. When transfected into COS cells two classes of receptor with affinity constants comparable to those on the parent cells were expressed.

Analysis of the structure of the IL-1 receptor indicates that though the size of the 80 kDa molecule is not altered under reducing conditions breaking of a disulphide bond may lead to loss of binding activity (Paganelli-Parker

and Kilian, 1988). A study by Speziale *et al* (1989) shows that the presence of high concentrations of lectins affects the ability of IL-1 to bind to its receptor, suggesting that carbohydrate moieties may be important for ligand interactions. Further reports indicate slight differences in size of the IL-1R seen in some instances (Bird and Saklatvala, 1987) may be due to alterations in carbohydrate levels on the protein. Mancilla *et al* (1989) suggest that the lectin effect differs according to cell type. Solari (1990) has shown two receptor forms on D10 cells of 80 and 60 kDa that have comparable peptide cleavage maps but may have different glycosylation patterns. These forms can also be distinguished by their preferential binding characteristics for IL-1 alpha and beta. Savage *et al* (1989) also show, using antibody recognition, that a 73 kDa receptor on their D10 line is related to the 80 kDa molecule.

1.5.c. The 60 kDa interleukin 1 receptor

The 60 kDa receptor was first described by Matsushima *et al* (1986a) on Epstein Barr virus transformed B cells, it was shown that more IL-1 alpha than beta was required to inhibit labelled IL-1 beta binding. Further reports have shown that cells with this receptor preferentially bind IL-1 beta (Scapigliati *et al*, 1989), but at a lower affinity than that seen for the 80 kDa receptor (Horuk *et al*, 1987). This lower affinity is thought to be due to a combination of a lower rate of association and a higher rate of dissociation of ligand (Horuk and McCubrey, 1989). The two receptor forms were compared using IL-1 analogues, and were shown to have different binding profiles (Horuk *et al*, 1987). By binding IL-1 alpha to B cell lines Bensimon *et al* (1989b) have shown the presence of a 68-72 kDa receptor band that can be separated into a high and a low affinity component.

The B cell receptor is glycosylated and treatment with endoglycosidase F reduces its molecular weight to approximately 48 kDa (Horuk and McCubrey, 1989). This form of IL-1 receptor is thought to be a distinct gene product as antibodies specific for the T cell receptor do not recognise the smaller molecule (Chizzonite *et al*, 1989). The mRNA for the 80 kDa receptor is also

not found in B cells by Northern analysis or S1 nuclease protection (Bomsztyk *et al*, 1989).

A recent study by Benjamin and Dower (1990) suggests that on the majority of EBV transformed B cell lines two classes of receptor exist, these show differential affinities for IL-1 alpha but bind IL-1 beta with the same affinity. Two molecular weight factors can also be demonstrated at 80 and 70 kDa. The EBV positive Burkitt lymphoma, Raji line is proposed to represent a second type of B cell that expresses only the 70 kDa factor and is unable to bind IL-1 alpha (Benjamin *et al*, 1990). In the case of the insulin receptor two molecules with different affinities have been described, that are produced as a result of alternative splicing of the same mRNA (Mosthaf *et al*, 1990).

1.5.d. Interleukin 1 receptor expression

The IL-1 receptor molecule is present at variable levels depending on cell type (Sipe, 1989). On T lymphocytes expression of IL-1R has been demonstrated on $CD4^+$ cells (Lowenthal and MacDonald, 1987), levels can be increased by stimulation with concanavalin A, with similar kinetics to the IL-2 receptor (Shirakawa *et al*, 1987; Dower and Urdal, 1987). The IL-1 receptor has also been shown on $CD8^+$ cytotoxic T lymphocytes (Klarnet *et al*, 1989). Recent reports describe variable receptor expression on the D10 cell line that can be modified by exogenous stimuli or ageing of the cells (Solari, 1990; Savage *et al*, 1989). In the case of the B cell receptor, glucocorticoids have been shown to increase the number of binding sites but not to affect affinity (Akahoshi *et al*, 1988).

It has often been noted that the affinity constant of the described receptor forms is several orders of magnitude above the concentration of IL-1 required to elicit a biological response (Savage *et al*, 1989). However some groups have detected higher affinity receptors and suggest these are responsible for mediating IL-1 function. Chin *et al* (1987) have shown that receptors on human lung fibroblasts bind IL-1 with dissociation constants in the picomolar range. Lowenthal and MacDonald (1986) demonstrated two classes

of IL-1 binding on EL-4 cells one has higher affinity and comprises 1-2% of the total receptor number. It has been suggested that the appearance of this form may be due to conformational changes in the receptor (Dinarello *et al*, 1989). It is possible that such high affinity receptors cannot be confidently identified by conventional Scatchard analysis.

1.5.e. Proteins associated with the IL-1 receptor

High molecular weight cross-linked bands at about 140 and 120 kDa have been demonstrated by various groups (Martin *et al*, 1988; Bird *et al*, 1987; Dower *et al*, 1985). The former may represent a complex between the 80 kDa characterised receptor and a 40 kDa moiety described by Kroggel *et al* (1988), while the latter may include a 26-30 kDa molecule (Bron and MacDonald, 1987; Savage *et al*, 1989). The 30 kDa band has been partially characterised and increased levels are seen after treatment of D10 cells with lectin, it may in some way stabilise the receptor within the membrane. This data has been used to describe a two chain IL-1 receptor in which the smaller molecule transduces the cellular signal. A recent report by Lewis *et al* (1990) using anti IL-1 receptor antibodies identifies components at 45, 50 and 68 kDa that associate with the 80 kDa receptor. An EL-4 cell variant has been identified that can bind IL-1 but is defective in its ability to internalise (Von Hoegen *et al*, 1989). This data has been used to suggest the need for a second factor to achieve ligand internalisation in these cells (Lewis *et al*, 1990).

1.5.f. Interleukin 1 signal transduction

There are several theories concerning the mechanism by which the IL-1 receptor binding event transduces a signal to the nucleus. It has been shown that binding of IL-1 to the 80 kDa molecule results in rapid internalisation of ligand (Bird and Saklatvala, 1987). It appears that some receptor-bound IL-1 is degraded in the lysosomes (Matsushima *et al*, 1986d; Quarnstrom *et al*, 1988) while a fraction can be located in an intact form at the nucleus (Mizel *et al*, 1987; Curtis *et el*, 1990). Interleukin 1 receptors are present in intact isolated nuclei (Grenfell *et al*, 1989).

To account for the more rapid effects of the cell in response to IL-1 various mechanisms have been suggested. Rosoff *et al* (1988) propose a novel system in which diacylglycerol is produced in the absence of phosphatidylinositol mobilisation. Other studies suggest adenylate cyclase activation (Chedid *et al*, 1989) or receptor phosphorylation (Gallis *et al*, 1989) as potential second messenger systems. Phosphorylation of cellular proteins in response to IL-1 has been observed (Kaur and Saklatvala, 1988). A triad of 27 kDa factors that label with ^{32}P at serine or threonine residues following IL-1 stimulation, has been demonstrated. Transmodulation of epidermal growth factor receptors has also been shown in response to IL-1, this is again thought to be dependent on phosphorylation of the receptor itself, but not to act via protein kinase C activation (Bird and Saklatvala, 1989).

In the case of the 60 kDa receptor there is little apparent internalisation (Horuk *et al*, 1987), but a transient translocation of protein kinase C to the membrane has been noted (Bomsztyk *et al*, 1989).

1.6. MODULATORS OF INTERLEUKIN 1 ACTION

It has long been known that factors present in biological fluids are able to affect the *in vitro* functions of IL-1. Initial reports noted that LAF activity in serum could only be detected when samples were at high dilution (Wood *et al*, 1983b) and that the levels of these factors could be influenced by the immunological condition of the host (Dinarello *et al*, 1981). More recently specific IL-1 inhibitors have been identified and characterised to varying degrees. These inhibitors could act at several levels: they can bind IL-1 itself and prevent receptor interaction, act as specific receptor antagonists, or alter the response of the cell to ligand binding (Larrick, 1989).

1.6.a. Interleukin 1 inhibitors

One IL-1 inhibitor has been particularly well characterised and recently cloned. The molecule was first identified by two groups, Arend *et al* (1985)

demonstrated that monocytes stimulated by PMA or immune complexes produced a 22 kDa factor able to inhibit IL-1 action in the LAF assay and on articular chondrocytes. A factor of comparable molecular weight was demonstrated in the urine of febrile patients (Seckinger *et al*, 1987b) and was able to prevent the production of PGE_2 and collagenase in response to both IL-1 molecules. The inhibitor was further shown to compete with iodinated IL-1 for cell binding sites suggesting a direct interaction with the receptor molecule (Seckinger *et al*, 1987a). Kinetics of production of the inhibitor suggest that release may be induced during monocyte differentiation (Roux-Lombard *et al*, 1989).

The inhibitor has been purified and shown to be present as three forms with similar N-terminal amino acid sequence but differing in their degrees of glycosylation (Hannum *et al*, 1990). The factor binds to the 80 kDa IL-1 receptor with a similar affinity to the ligand but does not compete for binding on a B cell line. Eisenberg *et al* (1990) isolated the inhibitor cDNA and showed that it encoded a protein with a classical signal sequence and was processed to a 17.1 kDa mature protein. When expressed in *E. coli* the inhibitor had similar mobility to the non-glycosylated, purified form and blocked IL-1 activity in the fibroblast assay. Sequence comparison with both IL-1s shows 26% homology with IL-1 beta and 19% with IL-1 alpha. A similarity in hydrophobicity plots is seen with IL-1 beta at the C-terminal region. The same inhibitor has also been cloned from the myelomonocytic line U937 and expressed in *E coli* (Carter *et al*, 1990). This molecule has been shown to inhibit the *in vivo* IL-1 effects on corticosteroid induction and neutropenia.

Another glycoprotein, uromodulin, isolated from the urine of pregnant women has been shown to be inhibitory in the thymocyte proliferation assay (Brown *et al*, 1986). The 85 kDa molecule (related to Tamm-Horsfall protein) was found to act by binding to both IL-1 and lectin (Muchmore and Decker, 1987). Due to its selective localisation in the kidney uromodulin has been suggested to play a role in the renal regulation of circulating IL-1 activity (Hession *et al*, 1987).

Brown and Rosenstreich (1987) described a 30-35 kDa moiety also derived

from human urine that was able to inhibit IL-1 action in the LAF assay, but was inactive in other assays (Korn *et al*, 1987). The protein was found to have sequence homology with DNAse 1 (Rosenstreich *et al*, 1988), and acted by releasing unlabelled thymidine from dead cells effectively diluting the tracer (Svenson and Bendtzen, 1989).

A synthetic peptide with homology to the envelope proteins of a number of viruses has been shown to inhibit monocyte cytotoxicity, studies indicate that the effects of IL-1 were blocked. The peptide inhibits IL-1 in the LAF assay and on the D10 line (Kleinerman *et al*, 1987), and has been demonstrated to prevent signal transduction by the IL-1 receptor (Gottlieb *et al*, 1989).

Other less well-characterised inhibitors have been described, most have been identified by the thymocyte co-mitogenic assay, the effects seen may therefore be a result of non-specific interactions with the lectin or other cytokines known to be active in the system. Monocytes activated with cytomegalovirus and B cell lines have been reported to produce a 95 kDa inhibitor (Rodgers *et al*, 1985; Scala *et al*, 1984; Roberts *et al*, 1986). LAF inhibitors have also been produced by epidermal cells and neutrophils in culture (Schwarz *et al*, 1987; Tiku *et al*, 1986).

The effects of IL-1 are also subject to modulation by other cytokines, the best characterised is TGF beta that blocks IL-1 action *in vitro* in the thymocyte proliferation assay (Wahl *et al*, 1988).

1.6.b. Soluble receptor molecules

In a variety of cases the surface receptor for a particular ligand has been identified in soluble form. Different cellular mechanisms result in release of these molecules. In some cases separate genes encode the surface and soluble receptors (Kress *et al*, 1983). Other instances describe alternatively spliced mRNA, in the case of the N-CAM adhesion molecule an in-frame stop codon is introduced that terminates the coding sequence before the transmembrane segment (Gower *et al*, 1988). The major histocompatability Qa-2 antigen and CD16 have an anchoring phospholipid tail that is subsequently cleaved (Stroynowski *et al*, 1987; Huizinga *et al*, 1988). The IL-2 receptor

however is solubilised by proteolytic cleavage at the cell surface (Robb and Kutny, 1987).

Soluble receptors have been suggested to serve a regulatory role, they may bind to and inhibit the action of the ligand as is the case with the IL-2 receptor (Kondo *et al*, 1988; Symons *et al*, 1988), or serve as carrier molecules.

Soluble receptors have now been described for a number of cytokines including IL-6 and interferon gamma (Novick *et al*, 1989), IL-4 (Mosley *et al*, 1989), TNF (Schall *et al*, 1990) and IL-7 (Goodwin *et al*, 1990).

A soluble form of the 80 kDa IL-1 receptor has not yet been identified, but by constructing a cDNA of residues 1-316 a truncated receptor has been expressed. This receptor binds IL-1 alpha in a comparable manner to the intact molecule (Dower *et al*, 1989), and has IL-1 inhibitory activity in an *in vitro* B cell proliferation assay (Maliszewski *et al*, 1990) and an *in vivo* allograft rejection system (Fanslow *et al*, 1990).

1.6.c. Circulating autoantibodies

The presence of autoantibodies to cytokines in normal individuals has been discussed by Bendtzen *et al* (1990) in a recent review. They have been implicated to occur commonly at low levels and may have a carrier function protecting the molecule from degradation and elimination, or serve as inhibitory factors to control cytokine effects. Pharmacological administration of an anti-interferon/interferon complex resulted in reduced systemic clearance while not affecting *in vitro* anti-viral activity (Rosenblum *et al*, 1985). Autoantibodies to TNF and lymphotoxin have been identified in the serum of healthy individuals and laboratory animals (Jeffes *et al*, 1989) suggesting that they are a normal phenomenon. Higher levels of anti-TNF antibodies have also been associated with infections and inflammatory disease (Fomsgaard *et al*, 1989). Other cases of autoantibodies to cytokines had previously only been seen in disease states (Panem *et al*, 1982; Bost *et al*, 1988) or when the immunogen had been administered in therapeutic trials (Vallbracht *et al*, 1981).

A high molecular weight IL-1 alpha binding factor present in human sera was investigated and found to be due to circulating autoantibodies. Further investigation has shown these to be present in the sera of 10% of normal individuals (Svenson *et al*, 1989), they have higher affinity than some cell surface IL-1 receptors and can compete in ligand binding assays.

1.6.d. Alpha$_2$ macroglobulin

Alpha$_2$ macroglobulin (alpha$_2$M) is a common molecule in serum known to bind many circulating proteins and has various immunomodulatory effects. The protein is composed of four subunits and undergoes a conformational change on entrapment of ligand. This may serve to expose a hydrophobic region that can interact with macrophages and hepatocytes and facilitate uptake from the circulation (Feldman *et al*, 1985).

Cytokine interactions with alpha$_2$M have recently been reviewed (James, 1990). An early report showed that platelet-derived growth factor was able to interact with plasma alpha$_2$M (Huang *et al*, 1985). In the case of basic fibroblast growth factor alpha$_2$M appears to bind covalently and inactivate the molecule, and may promote its removal from the circulation (Dennis *et al*, 1989). However transforming growth factor beta (TGF beta) binds in two ways to alpha$_2$M: covalently and as a latent, acid removable form (Huang *et al*, 1988) that may retain biological activity (O' Conner-McCourt and Wakefield, 1987). Alpha$_2$M is thought to act as a carrier molecule for serum IL-6 and complex formation does not inhibit IL-6 biological activity but makes the cytokine more resistant to proteolytic degradation (Matsuda *et al*, 1989).

Two groups have investigated the binding of IL-1 to alpha$_2$M, Borth and Luger (1989) demonstrated labelled IL-1 binding to a high molecular weight moiety in human plasma that could be precipitated by antibodies to alpha$_2$M. Teodorescu *et al* (1988) showed that IL-1 bound covalently and non-covalently to alpha$_2$M and that the complex retained bioactivity.

1.7. THE PHYSIOLOGICAL AND PATHOLOGICAL ROLES OF IL-1

The importance of IL-1 in eliciting an effective immune response and in

mediating a local inflammatory reaction have been previously discussed, however IL-1 may also be involved in normal physiological processes as is indicated by its presence in various *ex vivo* biological fluids. Endogenous pyrogen activity has been shown to be raised in the plasma of normal subjects following exercise (Cannon and Kluger, 1983), and in women after ovulation (Cannon and Dinarello, 1985). Interleukin 1 bioactivity has also been demonstrated in amniotic fluid, indicative of a role in reproduction (Romero *et al*, 1989). A role for IL-1 in systems such as normal bone turnover and sleep processes have also been suggested (Gowen *et al*, 1983; Dinarello, 1989).

The protective roles of IL-1 in response to acute infection have been discussed. A chronic disease state or autoimmune reaction may result from abnormal regulation of these host defence mechanisms. The inflammatory processes associated with local IL-1 release are themselves damaging to host tissue, if the subsequent repair process is inefficient or if cytokine production is dysregulated severe tissue damage may occur, as seen in conditions such as rheumatoid arthritis (Duff *et al*, 1988b).

The postulated role of IL-1 in a number of destructive disease processes, such as diabetes, kidney inflammation and neurological disease has been summarised by di Giovine and Duff (1990). Many of the *in vitro* properties of IL-1 suggest that it may be produced at high concentrations in the joint and contribute to the destructive process seen in rheumatoid arthritis.

1.7.a. Interleukin 1 in rheumatoid arthritis

Rheumatoid arthritis (RA) is a disease with both local and systemic manifestations. The disease is characterised by inflammation of the synovial membrane that lines the joint space, This leads to proliferation of synovium and cellular infiltration. The synovium then invades articular cartilage and subchondral bone, resulting in the joint destruction that is characteristic of RA (Henderson *et al*, 1987). The disease also has many systemic manifestations: an acute phase response is often seen in acute flares and

muscle wasting, weight loss and depression of cell-mediated immunity are typical (Duff, 1989). An overall activation of the immune system in RA is indicated by the presence of autoantibodies in the serum and joint exudate and raised levels of various soluble surface protein and receptor molecules released following cellular activation, such as the soluble IL-2R and CD8 (Wood *et al*, 1988; Symons *et al*, 1990).

Many of the actions of IL-1 indicate that it may play a role in the disease process of rheumatoid arthritis. Porcine catabolin was initially identified by its ability to induce cartilage resorption (Saklatvala, 1981), and it was later demonstrated that this was due to IL-1's stimulatory effect on chondrocytes resulting in the secretion of collagenase and proteoglycanase (Gowen *et al*, 1984). Synovial cells also produce prostaglandins and reactive oxygen species in response to IL-1, resulting in further inflammation and tissue damage (Dinarello, 1986). Additionally IL-1 has bone resorbing activity *in vitro* (Gowen *et al*, 1983). Intra-articular injection of IL-1 results in leukocyte infiltration into the joint space and the breakdown of articular cartilage (Pettipher *et al*, 1986). Many of the systemic disturbances seen in RA such as fever, the acute phase response and immune activation could also be attributed to IL-1.

Further evidence for a role of IL-1 in RA is its presence in inflammatory joint effusions. The measurement of IL-1 in biological fluids is difficult as bioassays may be non-specific and sensitive to any inhibitors present. A number of reports, however, have identified and partially characterised IL-1 bioactivity in synovial exudates (Wood *et al*, 1983a; Nouri *et al*, 1984; Fontana *et al*, 1982). Interleukin 1 has also been shown to be released by disaggregated synovial tissue into culture medium (Wood *et al*, 1985). Since specific immunoassays have become available high levels of both forms of IL-1 have been detected in RA synovial fluid (Symons *et al*, 1989; di Giovine *et al*, 1990), and in a double knee study IL-1 beta levels have been shown to be higher in the fluid from an inflamed joint when compared to a less inflammed or uninvolved contralateral joint (Rooney *et al*, in press).

Interleukin 1 mRNA levels in synovial tissue have been studied. In

cultured disaggregated tissue IL-1 alpha mRNA levels were higher than beta, but expression was more transient (Buchan *et al*, 1988). Using *in situ* hybridisation on synovial sections IL-1 beta mRNA appears most abundantly and is commonly expressed by cells staining with macrophage markers (Duff *et al*, 1988a; Firestein *et al*, 1990).

1.7.b. Measurement of circulating IL-1 levels

For some time it has been evident that serum or plasma contains many inhibitory factors that prevent detection of IL-1 by bioassay. Detection of circulating IL-1 therefore necessitated prior fractionation. This often resulted in the demonstration of activity at higher and lower molecular weights than predicted for IL-1 (Dinarello *et al*, 1984). The availability of immunoassays has allowed more reliable detection of blood IL-1 levels, though some of the protein may be in the biologically inactive precursor form. Factors also appear to be present in plasma that can mask IL-1 immunoreactivity in some assays (Capper *et al*, 1990; Cannon *et al*, 1988).

Many studies of IL-1 production in normal and disease situations have therefore used isolated peripheral blood mononuclear cells, to determine levels of synthesis and *ex vivo* activation state. These investigations have identified two distinct populations, that are described as high and low producers respectively (Molvig *et al*, 1988). The intersubject variability of IL-1 production in response to LPS has been described as extending over two orders of magnitude (Endres *et al*, 1989), and can be attributed in part to to the MHC class II haplotype expressed by the individual (Santamaria *et al*, 1989).

Studies concerning the rate of clearance of IL-1 using animal models showed that IL-1 was primarily cleared by the kidney (Kampschmidt and Jones, 1985). More recent work with radiolabelled IL-1 beta showed biphasic clearance from the blood with half lives of 3 minutes and 4 hours respectively (Klapproth *et al*, 1989). Circulating IL-1 was seen to be associated with the plasma fraction (Newton *et al*, 1988) and the route of clearance was again found to be the kidney, significant amounts of IL-1 were

also found to localise in the liver and intestine.

1.8. STUDY AIMS

Interleukin 1 is a factor with an important role in the mobilisation of an efficient immune response through both a systemic and a localised inflammatory action. It may however have destructive potential if the controls regulating its production or action are ineffective.

The aim of this study was to identify circulating IL-1 present in both normal and disease states. In particular, levels in rheumatoid arthritis were examined, in an attempt to assess further the role of IL-1 in the pathogenetic process. We also hoped to define any factors that associated with the molecule in plasma and assess their ability to modify or inhibit IL-1 bioactivity.

As an approach to modulating the potentially-damaging effects of IL-1 *in vivo* we also tried to identify and characterise cellular enzymes responsible for processing the beta form. The aim was to block enzyme action using synthetic peptides designed around the cleavage site of the IL-1 beta propeptide, and thus prevent either release from the cell or the surface/ extracellular processing to the active (17 kDa) form.

2. METHODS

2.1. CELL CULTURE

2.1.a. Culture conditions

Continuous cell lines and primary cultures were maintained in RPMI 1640 (Northumbria Biologicals Ltd (NBL), Northumberland), which was supplied as a 10x concentrated solution. The stock was diluted with sterile distilled water (Travenol; Baxter, Norfolk) and sodium bicarbonate added as a buffering agent (2.2g/l; NBL), the pH was then adjusted to 7.4 with sodium hydroxide (1N; NBL). Before use, medium was supplemented with 5 or 10% heat inactivated (56° C for 30 minutes) foetal calf serum (FCS, NBL), glutamine (2mM; NBL) and penicillin/streptomycin (100U/ml; NBL).

Cell lines were routinely cultured in $75cm^2$ flasks (Costar, NBL) and passaged as required. Growth was maintained at 37° C in a humidified atmosphere with 5% CO_2. Culture supernatants were regularly tested for the presence of mycoplasma (Section 9.1.a.).

2.1.b. Interleukin 1 bioassay

A subclone of the mouse thymoma EL-4 cell line originally described by Gearing *et al*, (1987) was used for IL-1 assay. The line EL-4.NOB-1 when stimulated by IL-1 responds by producing IL-2, which can be measured by its ability to maintain the viability of the IL-2 dependent cytotoxic T cell line CTLL-2. The NOB-1 line was routinely grown in RPMI with 5% FCS and passaged regularly to keep cultures at a low density. If allowed to reach high density cells start to secrete IL-2 spontaneously. Growth in low serum medium (1% FCS) for one week was used to restore inducible production. CTLL-2 cells were grown in media containing 5% FCS and supplemented with 5% crude T-cell growth factor (TCGF) prepared from rat spleen cell culture (Section 9.1.b.). Cultures were re-fed with TCGF every 2-3 days.

For assay, EL-4 cells were washed and resuspended in fresh medium at a density of $2x10^6$/ml and 100ul seeded in 96 well plates (NBL). Human recombinant IL-1 alpha or IL-1 beta (National Institute for Biological Standards and Control (NIBSC), Hertfordshire) was used as a standard curve at concentrations between 1ng/ml and 100fg/ml. Samples were tested in triplicate

at 10%, 1% and 0.1%. One hundred microlitres of standard, sample, or media as a negative control, was added to appropriate wells and the plate incubated for 24 hours.

Culture supernatants were then harvested, 50ul was transferred from each well into a duplicate 96 well plate. CTLL-2 cells were resuspended at $1x10^5$/ml in fresh media and 50ul was added to each well. Rat TCGF at 5% was used as a positive control, and media alone as negative control. After 20 hours of culture, viability of the CTLL-2 cells was measured using a colourimetric assay first described by Mosmann (1983). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoluim bromide (MTT; Sigma, Dorset), is a yellow substrate that is converted to a blue formazan product by mitochondrial enzymes. Ten microlitres of MTT (5mg/ml in phosphate buffered saline (PBS, Section 9.3.a.)), was added to each well for 4 hours, the formazan was then solubilised by addition of 100ul 0.04N hydrochloric acid (HCl; BDH) in isopropanol (Sigma). This was measured as optical density (OD) by scanning plates at 570-630nm using a Dynatech MR-700 microplate reader.

2.1.c. Anti IL-1 antiserum

Specificity of the IL-1 bioassay was assessed using sheep antisera (NIBSC) raised against recombinant human IL-1 alpha or IL-1 beta. Antisera were screened by incubation for 2 hours at room temperature with standard IL-1 preparations prior to EL-4 assay. A titre of 1 in 1000 was found specifically to inhibit 100pg/ml of IL-1 activity. In further experiments antibody at this titre was used to neutralise IL-1 in biological samples.

2.1.d. IL-1 inhibitor assay

To test for specific inhibitors of IL-1 activity, the sample was pre-incubated with either IL-1 alpha or IL-1 beta (at 10pg/ml) for 2 hours at room temperature. EL-4.NOB-1 cells were then added and the assay continued as described. Viability of EL-4 and CTLL-2 cells after culture was tested by adding 0.5% trypan blue (Sigma) in PBS.

2.1.e. Cellular production of IL-1 beta

Various human monocytic cell lines were tested for their ability to produce IL-1 beta propeptide and to process it to the mature 17kd form. HL-60 and U937 lines were cultured in RPMI-1640 with 5% FCS and passaged every 2-3 days, THP-1 cells required 10% FCS and media supplemented with $2x10^{-7}$M 2-mercaptoethanol (BDH, Glasgow).

The stimuli used for activation were LPS, from (*E. coli*) serotype 0127:B8 (100ng/ml and 10ug/ml; Sigma), zymosan from *Saccharomyces cerevisiae* (1mg/ml; Sigma), and PMA (0.1ug/ml and 1ug/ml; Sigma). Cultures were separated into supernatant and cellular fractions, cells were lysed using 1% sodium dodecyl sulphate (SDS, BDH) with protease inhibitors (Aprotinin, from bovine lung, 0.67U/ml; Sigma, and Pepstatin A, 10ug/ml; Sigma) and processed for Western blotting (Section 2.9.a.).

2.1.f. Protease inhibitor effects

THP-1 cells were found to produce 31kd IL-1 beta and to process it to the mature 17kd form, this line was used to examine the effects of protease inhibitors. Various protease inhibitory factors were tested; aprotinin was added at 0.67U/ml, Pepstatin A at 10ug/ml, phenylmethylsulphonylfluoride at 1mM (PMSF, Sigma) and leupeptin at 10ug/ml (from *Streptomyces* species, Boehringer Mannheim, Lewes). 3,4-Dichloroisocoumarin (3,4-DC, Boehringer Mannheim) and 1,10-phenanthroline (1,10-PA, Sigma) were found to be toxic at high levels and so final concentrations of 100 and 10ng/ml were used. Inhibitors were added to cells at the start of culture in serum free medium with 0.1ug/ml PMA to stimulate IL-1 production. After 24 hours stimulation, supernatants were removed, the cells were lysed as described, and 31 kDa and 17 kDa levels of IL-1 beta were measured by Western blotting under reducing conditions (Section 2.9.a.). To assess mRNA levels cell preparations were made as described for slot blot analysis (Section 2.8.b.).

2.2. INTERLEUKIN 1 BETA PROCESSING

2.2.a. Processing of cell lysate IL-1 beta

Inhibition of processing was tested in a cell lysate system. Pro IL-1 beta was produced by stimulating $1x10^6$ THP-1 cells/ml with 0.1ug/ml PMA for 24 hours. Culture supernatant was then removed and the cells sonicated for four 30 second bursts at a frequency of 8 microns, on ice in fresh, serum free media at $1x10^7$ cells/ml. The nuclei were removed by centrifugation for 10 minutes at 400xg, membranes were separated by a second spin at 48000xg for 30 minutes. The supernatant was removed and stored at -20° C, the pellet was resuspended at $1x10^7$ cell equivalents/ml and frozen. Cell lysate and membrane preparations were used for processing activity, preparations were only thawed once before use.

For inhibition experiments crude 31 kDa propeptide was added to an equal volume of unstimulated cell lysate, membranes or fresh cells at $1x10^6$/ml. The processing reaction was incubated at 37° C for 1, 2, 6, 12 and 24 hours and then frozen, production of mature 17 kDa IL-1 beta was assessed by Western blotting under reducing conditions (Section 2.9.a.).

2.2.b. Processing of recombinant pro IL-1 beta

A recombinant form of human IL-1 beta propeptide was also used in processing experiments. The 31 kDa precursor was produced in *E. Coli* and was obtained as a partially purified solution at 55ug/ml in 100mM Tris-HCL with 1mM sodium azide, from Glaxo Group Research, Greenford. Preparations of cell lysate, membranes and supernatants were produced at concentrations of $1x10^7$ cell equivalents/ml, as described (Section 2.2.a.). Cells were also fractionated after stimulation with 100ng/ml LPS or PMA for 2 hours. Some membrane preparations were heated to 50° C for 10 minutes to determine the temperature sensitivity of the processing activity.

Processing experiments were carried out by incubating 5ul of recombinant propeptide with 10ul of the different cell fractions, or tissue culture medium as a negative control, for 2 hours at 37° C. The reaction was stopped by freezing to -20° C. Cell lysates, membranes and supernatants were all

tested for intrinsic IL-1 immunoreactivity after a comparable incubation time.

2.2.c. Time course of IL-1 beta propeptide processing

Recombinant IL-1 beta propeptide was incubated with medium alone, THP-1 cell lysates or membrane preparations. Aliquots of the reaction were removed immediately, and after 15 min, 30 min, 45 min and then 1, 1.5, 2, 2.5, 3, 4 and 5 hours. The aliquots were frozen immediately and analysed by Western blotting under reducing conditions (Section 2.9.a.).

2.2.d. Inhibition of processing

Protease inhibition of processing was examined, the protease inhibitors previously described (Section 2.1.f.) were used at 1x and 10x the described concentrations, apart from 3,4-DC and 1,10-PA that were used at 1 and 10ug/ml. The elastase inhibitor elastatinal (Sigma) was also tested at a concentration of 1 and 10mM.

2.2.e. Synthetic peptides

Synthetic peptides were made by D. MacLean from the University of Edinburgh Department of Chemistry using an Applied Biosystems 430 A Peptide Synthesiser. They were purified by an initial gel filtration step, followed by an ion exchange column and finally reverse phase HPLC. The product gave a single peak by analytical HPLC and a single band by isoelectric focusing. Peptides were designed from the sequence around the cleavage site of pro IL-1 beta. Peptide A consisted of amino acids 102-138 of the precursor molecule, peptide B residues 111-122, and peptide C was composed of a sequence on the N terminal side of the processing site at residues 101-112. Peptides were freshly made up to a 10^{-3} molar solution in dimethyl sulphoxide (DMSO, Sigma) and diluted to 10^{-5}-10^{-7}M with serum free medium.

2.3. PREPARATION OF *EX-VIVO* SAMPLES

2.3.a. Blood samples

Blood samples for measurement of IL-1 immunoreactivity were taken immediately into 5ml potassium ethylenediamine tetra-acetic acid (K_3EDTA, $5x10^{-3}$ mol/l) tubes (Labco, Labsales) with 0.67U/ml aprotinin. Larger volume samples were taken into 50ml polypropylene tubes (NBL) also containing K_3 EDTA (Sigma) and aprotinin. When possible cells were spun down immediately at 200xg for 10 minutes, alternatively they were stored at 4° C until centrifugation. Platelets were then removed by a further spin for 10 minutes at 1000xg, 500ul aliquots of plasma were stored at -20° C.

To ensure that possible LPS contamination and storage of samples at 4° C was not affecting IL-1 measurement, aliquots of a single blood sample were incubated in collection tubes for various lengths of time between 0 and 4 hours. Similar tubes were spiked with 100ng/ml LPS and stored at 4° C for the same times before plasma preparation.

Large scale plasma samples for chromatographic separation were further treated by dialysis overnight against 10 volumes of the column starting buffer used. Dialysis tubing used had a molecular weight cut-off of 12-14 kDa (Gibco-BRL).

2.3.b. Synovial fluid

Synovial fluid was collected and immediately centrifuged to remove cellular material, samples were then pooled and stored at -70° C in 500ml volumes. On thawing 3ml of 0.5M Na_2EDTA (Sigma) was added to prevent clotting, hyaluronidase (Sigma) was added to a concentration of 100U/ml and incubated for 45 minutes at 37° C. Fluid was then dialysed against 5 litres of starting buffer for 24 hours, and centrifuged at 400xg to remove any precipitate.

2.3.c. Sample concentration

Small scale concentration, of up to 20ml, was done using Centrifugal Ultrafree filters (Millipore, Bedford) with 30 kDa molecular weight cut-off.

Centrifugal units were washed through with 20ml of distilled water before use. Twenty millilitre aliquots of sample were added to the filter cup unit which was then put onto the receiver unit and centrifuged at 2500xg for 30 minutes. The filter cup was removed and concentrate recovered by a further spin of the inverted unit into a second tube.

Large scale concentration, of samples of over 1 litre used a Minitan Tangential-Flow system (Millipore). Sample was circulated across four polysulphone filter plates of 30 kDa cut-off. Plates were preconditioned by passing PBS through the system before use. Sample was fed into the unit, filtered at a back pressure of 10 psi and recirculated until the retentate volume was 50-100ml.

2.3.d. Plasma extraction

Extraction of plasma, first described by Cannon *et al* (1988), was found to increase the level of detectable IL-1 beta by immunoassay. Aliquots of plasma were therefore thawed and extracted twice; two volumes of chloroform (BDH) were added to one of plasma and shaken for 5 minutes. The two phases were separated by centrifugation at 10000xg for 10 minutes and the upper, aqueous phase removed. The extracted plasma was respun to remove any remaining precipitate and stored at -20° C before IL-1 assay.

2.3.e. Assessment of disease activity

Samples were taken from normal healthy controls and patients with definite or classical rheumatoid arthritis (Ropes *et al*, 1958) who were attending a rheumatology out patient department, or had been admitted to a rheumatology ward. The patient group was assessed for a number of measures of disease activity including Ritchie joint index (Ritchie *et al*, 1968), duration of early morning stiffness, and visual analogue pain score.

Corresponding patient blood samples were taken to test standard laboratory measures of disease activity such as haemoglobin concentration (Hb), white cell count (WCC), platelet count (Plt), erythrocyte sedimentation rate (ESR), and rheumatoid factor titre (RhF).

2.3.f. Patient drug treatments

All patients were receiving non-steroidal anti-inflammatory drugs, and some also received second line therapy. Hospitalised patients studied longitudinally all had either oral or intra-articular steroids, three received oral or injectable gold, and one longitudinal and two out-patients were given anti-malarials.

2.3.g. Statistical analysis

Comparison of mean values were analysed using Students *t*-test, and correlations assessed with the Spearman rank correlation coefficient.

2.4. INTERLEUKIN 1 IMMUNOASSAYS

2.4.a. IL-1 beta Enzyme-Linked Immunosorbent Assay (ELISA)

Two IL-1 beta ELISA's were used; one commercially available from Cistron Biotechnology (Laboratory Impex, Middlesex) and the other using reagents kindley supplied by Roussel UCLAF (Romainville).

2.4.a.i. Cistron IL-1 beta ELISA

The assay is a four stage test in which a monoclonal antibody specific for IL-1 beta is supplied coated onto a 96 well microtitration plate. To this was added 100ul of either standards or samples in duplicate; the IL-1 beta preparation supplied was reconstituted with deionised water to 50ng/ml and diluted in the buffer provided to give a range of concentrations between 1000pg/ml and 20pg/ml. Samples were diluted in buffer if necessary. The plate was then covered and incubated for 2 hours at 37° C. Wells were then washed three times, using the solution provided which was diluted with 400ml of water. Excess buffer was removed and secondary antibody, polyclonal rabbit anti-human IL-1 beta reconstituted in deionised water, was added for a further 2 hours. Following a second wash, horseradish-peroxidase (HRP) labelled anti-rabbit immunoglobulin G (IgG), in assay buffer, was added and the plate incubated at room temperature for 30 minutes. The substrate was

prepared immediately before the final wash step, by dissolving the o-phenylenediamine (OPD) tablets provided in water, prior to use 3% hydrogen peroxide was added. One hundred microlitres of substrate was pipetted into each well and the reaction left at room temperature up to 15 minutes to develop. Sulphuric acid (50 ul, 4N; BDH) was added to stop the reaction and the colour intensity in the plate measured at 490nm using a microtiter plate reader.

2.4.a.ii. Roussel IL-1 beta ELISA

The Roussel IL-1 beta ELISA was used as described by Fontaine *et al* (1989). It was performed on Maxisorb microtiter plates (Nunc, Gibco-BRL). Plates were coated with 100ul of a 10ug/ml solution of a monoclonal anti-IL-1 beta in coating buffer (0.1M sodium carbonate, pH 9.6), at room temperature for 16-24 hours. Antibody solution was then removed and plates blocked with 200ul of 3% bovine serum albumin (BSA, Sigma A4503) in PBS for 30 minutes. The plate was washed three times in PBS with 0.1% polyoxyethylenesorbitan monolaurate (Tween 20, Sigma). Interleukin 1 beta standard was reconstituted to 100ug/ml with distilled water, and was further diluted in PBS with 0.1% bovine serum albumin (BSA) and 0.1% Tween 20 to give a concentration range between 2ng/ml and 20pg/ml. Samples and standards were added in duplicate at 50ul/well. At the same time 50ul monoclonal anti-IL-1 beta HRP conjugate (2ug/ml) was also added, and incubated at 4° C overnight. Substrate was made up just before use, one OPD substrate tablet (10mg; Sigma) was dissolved in substrate buffer (0.065M disodium hydrogen phosphate (Aldrich, Dorset), 0.017M citric acid monohydrate (Fisons, Loughborough), pH 6.3). The plate was washed, 3% hydrogen peroxide (Sigma) was added to the substrate and 100ul pipetted into each well. The reaction was incubated at room temperature in the dark for 15 minutes and stopped with 50ul of 4N sulphuric acid. Optical density was read at 490nm.

2.4.b. IL-1 alpha radioimmunoassay (RIA)

A commercially available specific IL-1 alpha RIA was obtained from

Amersham International plc. Before starting the assay all reagents were brought to room temperature, dilution buffer was heated to 40° C until the gel melted and was then made up to 50ml volume with deionised water. The assay was done in 5ml polypropylene tubes (Sarstedt, Leicester). Human recombinant IL-1 alpha was provided as a standard and was reconstituted with buffer, to a concentration of 320fmol/ml. This stock was further diluted to give a concentration range between 160fmol/ml and 5fmol/ml, 100ul of standard or sample was added to the tubes in duplicate. Rabbit anti-human IL-1 alpha antisera was reconstituted to 11ml with buffer, and 100ul was added to each tube, these were mixed, covered and incubated for 4 hours at room temperature. Iodinated IL-1 alpha was used as tracer, it was reconstituted with assay buffer and 100ul added to each tube. The assay was incubated at 4° C for 16-20 hours. Donkey anti-rabbit antisera was provided coated onto a magnetisable polymer, Amerlex M. Two hundred and fifty microlitres/tube was added, vortexed and incubated for 10 minutes at room temperature. The Amerlex-antibody complex was separated by centrifugation at 1500xg for 10 minutes at 4° C. The supernatant was decanted and tubes inverted on absorbent paper to drain excess fluid. The amount of ^{125}I IL-1 alpha present was determined by counting in a gamma counter for 60 seconds.

2.4.c. Assay validation

To ensure that immunoassays were suitable for measuring plasma IL-1, recovery of added protein and dilution characteristics were assessed; plasma had IL-1 standard added to give a final concentration of 200pg/ml both before and after extraction, percentage recovery was determined. Plasma with added IL-1 and untreated plasma were serially diluted before assay to confirm parallel dilution characteristics. Plasma from both normal and patient groups were tested in this manner.

Inter-assay variability was determined, and in some cases standard curves were prepared in a plasma sample known to have low endogenous IL-1, to control for potential plasma effects in the assay.

2.5. ANALYSIS OF PLASMA IL-1 BINDING PROTEINS

2.5.a. Gel filtration

Radiolabelled IL-1 was added to plasma to allow identification of any IL-1 binding proteins. For analysis of binding factors under physiological conditions 3ml volumes of unextracted or extracted (Section 2.3.d.) plasma had 3ng of ^{125}I IL-1 alpha or beta (specific activities 70-120uCi/ug and 126-253uCi/ug respectively; DuPont, Hertfordshire) added, these were incubated overnight at 4° C. Specificity of binding was determined by including a hundred fold excess of non-labelled IL-1 (Biogen SA) or TNF alpha (a gift from Dr Guenther Adolph, Ernst Boehringer Institut fur Arzneimittel, Austria) in the reaction. The molecular weight of any IL-1/protein complexes formed was examined by passing the incubation reaction down a gel filtration column in PBS buffer, and measuring radiolabel in the resulting fractions (Section 2.6.).

2.5.b. Covalent cross-linking

The presence of binding proteins was also analysed using covalent cross-linking techniques. Fifty microlitre aliquots of plasma samples or partially purified binding protein were incubated overnight at 4° C with 0.5ng of radiolabelled IL-1, with relevant cold competition controls. Reactions were treated with freshly prepared cross-linker for 30 minutes at 4° C. Various homobifunctional cross-linking reagents were used, all were prepared at 10mg/ml concentrations in DMSO and 5ul/reaction was added; disuccinimidyl suberate (DSS) and ethylene glycolbis(succinimidyl succinate) (EGS) were obtained from Pierce and Warriner (Cheshire). The cross-linking reaction was stopped by adding an equal volume of loading dye (Section 9.5), and samples were analysed by sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE, Section 2.9.a.), followed by autoradiography.

2.5.c. IL-1 receptor binding

A 3T3 fibroblast cell line was used for IL-1 receptor cross-linking studies. Cells were cultured in RPMI-1640 with 5% FCS, antibiotics and

glutamine, and grown as adherent monolayers. Cultures were maintained at a subconfluent level by passaging every 3-4 days. They were first washed in serum free medium then 1ml of 1x trypsin (NBL) was added to each $75cm^2$ flask until cells began to lift from the surface. To this 9ml of media with 5% serum was added, cells were centrifuged and the cultures divided 1:10 into fresh flasks.

To test the receptor binding activity of ^{125}I IL-1 alpha, cross-linking studies were performed on a 3T3 fibroblast line as described (Dower, 1985). Solutions used are described in Section 9.2. Monolayers of cells were washed in PBS and then incubated with 1ng/ml labelled IL-1, with or without 100 fold excess cold cytokine as described (Section 2.5.a.). Interleukin 1 was prepared in binding buffer and was added to cells for 4 hours at 8° C in a volume of 5ml. Cells were then washed three times in ice cold binding buffer to remove unbound label. A further 5ml of buffer was added with DSS at a concentration of 1mg/ml as cross-linker, this was left on ice for 45 minutes. Cross-linker was replaced by 5ml of quenching solution, and cells scraped off the flask using a rubber policeman. These were centrifuged at 200xg for 10 minutes at 4° C and lysed by resuspending the pellet in 100ul lysis buffer containing protease inhibitors. Samples were analysed by SDS-PAGE (Section 2.9.a.).

2.5.d. Characterisation of the IL-1 beta binding protein

2.5.d.i. Binding kinetics

The kinetics of binding of IL-1 beta to plasma proteins was studied by incubating iodinated IL-1 beta with plasma for various time periods. Binding was carried out at 4° C and room temperature for 0.5, 1, 1.5, 2, 2.5, and 3 hours. A control sample was incubated overnight. The reaction was stopped by cross-linking and adding SDS-PAGE loading buffer (Section 9.5.), samples were then stored at -20° C.

2.5.d.ii. Effect of reducing agents

To analyse the effect of reducing agents on plasma binding proteins

samples were prepared as described (Section 2.5.b.). Before loading on SDS-PAGE gels an equal volume of loading buffer and dithiothreitol ((DTT) 100mM, Sigma) was added. These were then heated at 100° C for 10 minutes.

2.5.d.iii. Temperature stability of IL-1 binding proteins

Temperature stability of the binding protein was determined by heating samples to 37° , 40° , 50° , 60° and 70° C for 10 minutes prior to incubation with ^{125}I IL-1 beta, and cross-linking. Samples had EDTA added to a concentration of 5mM to prevent clotting during treatment.

2.5.e. N-Glycanase treatment of IL-1 beta binding protein

Glycosylation of the partially purified protein was assessed by treatment with N-Glycosidase F (N-Glycanase) purified from *Flavobacterium meningoseptum* (Genzyme, Maidstone). Fifty microlitres of the ion exchange purified protein preparation (Section 2.7.b.) was incubated with IL-1 beta and cross-linked. The reaction was treated with 5ul of quencher (Section 9.2.), and boiled for 3 minutes in the presence of 0.5% SDS and 0.1M 2-mercaptoethanol. Twenty microlitres of this solution was made to 0.2M with sodium phosphate pH 8.6 (Section 9.3.a.), to 10mM with 1, 10 phenanthroline (diluted in methanol, BDH), and 1.25% with Nonidet P-40 (Sigma). N-Glycanase was added to a concentration of 10U/ml, in a total volume of 60ul. The reaction was incubated overnight at 37° C, and results analysed by SDS-PAGE (Section 2.9.a.).

2.6. PLASMA FRACTIONATION

To test for bioactive and immunoreactive IL-1 in plasma, extracted and unextracted (Section 2.3.d.) samples were fractionated by gel filtration before assay. A Sephacryl S-200 (Pharmacia-LKB Biotechnology, Buckinghamshire) matrix was used with a PBS buffer at pH 7.4 (Section 9.3.a.). The column was 100cm in length with an internal diameter of 16mm (Pharmacia), a flow rate of 1ml/minute was used and fractions were collected over 2 minutes. Twelve millilitres of sample were allowed to load onto the

column under gravity and proteins were then eluted. The protein concentration was determined in each sample by OD measurement at 280nm, fractions were stored at -20° C prior to use in IL-1 assays. The column was calibrated using standard gel filtration molecular weight markers ranging from 12-200 kDa, supplied in kit form (Sigma, MW-GF-200).

Plasma previously incubated with 500ng iodinated IL-1 alpha or beta was also analysed by gel filtration. Three millilitre samples were loaded onto the column under gravity and eluted in PBS. Fractions of 2ml were collected and the amount of radiolabel was measured by counting for 1 minute in a gamma counter. Results were expressed as a percentage of total counts. For one experiment IL-1 beta/protein complexes from gel filtration were pooled, cross-linked, concentrated, and analysed by SDS-PAGE (Sections 2.3.c. and 2.9.a.).

2.7. PURIFICATION OF IL-1 BETA BINDING PROTEIN

2.7.a. Wheat germ agglutinin column purification

Wheat germ Lectin-Sepharose 6MB (Pharmacia) was used for partial purification of the binding protein from plasma. A column of length 65mm and internal diameter 16mm was prepared with Wheat germ Sepharose in PBS. Twenty millilitres of plasma was dialysed overnight at 4° C in PBS and loaded onto the column at a flow rate of 4ml/hour. The column was washed in 2 volumes (40ml) of PBS/0.3M NaCl (Sigma) followed by PBS/10% (v/v) ethylene glycol (Sigma). Glycosylated proteins were eluted from the column with 2 volumes of PBS/500mM N-acetyl-glucosamine (Sigma)/0.3M NaCl, two millilitre fractions were collected from each step. The column was regenerated as described in Section 9.3.b.

Fractions were assessed for protein concentration by measurement of the OD at 280nm, and for the presence of binding protein (Section 2.5.b.).

2.7.b. Ion-exchange chromatography

For large scale purification of IL-1 beta binding protein from synovial fluid a DEAE Sephacel (Pharmacia) anion exchange column was used. Initial

experiments were done to establish binding and elution characteristics. Aliquots of 1.5ml of DEAE Sephacel were washed ten times in 10ml of 0.5M Tris-HCl (Sigma) each at a different pH, ranging from pH 5-9. They were then equilibrated to a lower salt concentration, by washing five times in 0.01M Tris-HCl, at the same pH as the previous step. One millilitre of synovial fluid, diluted 1:5 in the respective buffer, was added to each tube and mixed for 10 minutes. The supernatant was removed and assayed for binding protein. The lowest pH at which all binding activity had been removed from the fluid was selected as starting buffer.

Secondly, the salt concentration required to elute the protein was established. Aliquots of Sephacel were washed as described above, in 0.5M Tris-HCl, at the selected pH, and then in starting buffer. Gel was then equilibrated with a range of salt concentrations, between 0.01M and 0.3M, by washing as before. Sample was added and the supernatant assayed for binding activity, the lowest salt concentration in which activity was present was used, to determine the range of the elution gradient.

The DEAE Sephacel column used was 500mm in length and 50mm in diameter, and was equilibrated with 0.01M Tris-HCl at pH 5.5 (starting buffer). Synovial fluid was prepared as described (Section 2.3.b.), using a starting volume of 750ml/run. Sample was loaded at a flow rate of 1ml/minute at 8° C, and the column was washed overnight in starting buffer. Proteins were eluted with a continuous gradient of 0.01M-0.5M NaCl. Ten millilitre fractions were collected after a 250ml void volume had eluted, 1ml aliquots were taken for measurement binding activity, the remainder was stored at -70° C. The column was regenerated as described in Section 9.3.b. Pooled fractions containing binding protein were concentrated as described (Section 2.3.c.) before further purification steps.

2.7.c. IL-1 beta affinity chromatography

Concentrated eluate from the ion-exchange column was further purified by IL-1 beta affinity chromatography. The matrix was prepared using Activated Thiol Sepharose-4B (Pharmacia) which was obtained as a freeze dried powder.

The gel was hydrated before use (Section 9.3.b).

The ligand used was an altered form of human IL-1 beta with a substitution at position 138, where a lysine was replaced by a cysteine to facilitate binding to thiol groups. Protein was provided as a 3.44mg/ml solution in 100mM Tris-HCl, 1mM DTT and 1mM EDTA at pH 7.5 by the Glaxo Institute for Molecular Biology SA (Geneva).

Dithiothreitol was removed from the IL-1 prior to coupling by gel filtration over Sephadex G25 (Pharmacia) in 100mM sodium phosphate buffer at pH 8 (Section 9.3.a.). Fractions containing protein, as measured by OD at 280nm, were pooled and loaded immediately onto the activated Sepharose column. The resulting gel contained 5mg mutein IL-1/ml of matrix.

The concentrated binding protein sample was passed over the column twice and washed in twenty column volumes of starting buffer, followed by three high stringency washes (Section 9.3.a.). Binding protein was eluted with a 4ml pulse of 0.1M glycine (Sigma) at pH 3. The pH of fractions were tested, any that were acidic were neutralised immediately with 0.1N sodium hydroxide (NBL). The column was washed after use with Tris-HCl at pH 7.5. Fractions were tested for binding activity as described (Section 2.5.b.).

2.7.d. HPLC of IL-1 beta binding protein

The semi-purified protein preparation was concentrated by vacuum centrifugation and loaded onto a reverse phase, RP300 Aquapore 30 x 2.1mm C8 column (Applied Biosystems). Sample was eluted with a 10-70% acetonitrile gradient with 0.3% trifluoroacetic acid over 45 minutes, at a flow rate of 200ul/minute. Optical density was measured at 220nm and protein peaks were collected by hand. Individual peaks were analysed for ^{125}I IL-1 beta binding activity.

2.8 MOLECULAR BIOLOGY TECHNIQUES

All experiments were done using RNAse free techniques and reagents (Section 9.4.a.).

2.8.a. cDNA probe preparation

Cytoplasmic mRNA was detected using cDNA probes. An interleukin-1 beta cDNA encoding amino acids 5-269 was obtained from Dr D Carter (The Upjohn Company, Kalamazoo, MI). This was an insert in a pBR322 plasmid that had been transformed into *E. coli* HB101, and conferred tetracycline resistance. As a control for mRNA loading a 7B6 cDNA (Dr U Torelli, Univ Modena, Italy) coding for a cell cycle independent mRNA was used. This was propagated in *E coli* in pBR322 and comprised a 708 base pair fragment. It has previously been established by Northern analysis that the IL-1 beta cDNA probe binds to a 1.8kb mRNA species from stimulated peripheral blood mononuclear cells (E Thornton, personal communication).

Plasmid DNA was prepared using standard growth and alkaline lysis techniques as described by Sambrook *et al* (1989). The 7B6 DNA was prepared by E Thornton. Interleukin-1 beta cDNA was restricted using Pst I, and 7B6 with Pst I and Dra I enzymes (Gibco-BRL). Preparations were run on low melting point agarose gels and the required fragment cut out, following staining with ethidium bromide.

Probes were labelled from agarose gel preparations using a random primer oligolabelling reaction. The cDNA preparation was denatured by boiling for 10 minutes followed by incubation for at least 10 minutes at 37° C. One hundred nanograms of DNA was added to 10ul oligolabelling buffer (Section 9.4.b.), 2ul of 10mg/ml BSA and 50uCi of ^{32}P labelled dCTP (DuPont). One microlitre of large fragment DNA polymerase I (Klenow, Gibco-BRL) was then added and the volume made up to 50ul with water. The reaction was incubated overnight at room temperature and then stopped by addition of 200ul of 500ug/ml calf thymus DNA (Sigma). Probe was separated from free label by passing down a Sephadex G-50 (Pharmacia) column and eluted in Tris-EDTA pH 8.1 (TE, Section 9.4.b.). The column was monitored using a Geiger counter and the first radiolabelled peak collected. Specific activity of the probe was estimated by counting a 1ul aliquot, the reaction gave at least 10^7-10^8 cpm for 100ng of DNA added.

2.8.b. Slot blot analysis of mRNA

Slot blot analysis was performed according to the method of White and Bancroft (1982). Solutions used are described in Section 9.4.b.. Cell suspensions containing $1x10^6$ cells were centrifuged at 400xg and the pellet washed in PBS. The cells were then resuspended in 45ul ice cold TE and 5ul of 5% Nonidet P-40 was added. Preparations were mixed on ice for 5 minutes and a further 5ul of Nonidet was added. Nuclei were removed by spinning for 3 minutes at 1500xg, 50ul of the supernatant was taken and added to a 1.5ml tube containing 30ul of 20x saline sodium citrate (SSC) and 20ul of 37% (v/v) formaldehyde. This was heated to 60° C for 15 minutes, and then stored at -70° C if required. Samples were diluted 1:2, 1:5 and 1:25 with 15x SSC, relevant denatured cDNAs were prepared as hybridisation controls at concentrations between 0.1 and 0.001ng/ml. Cytoplasmic mRNA and control DNA were transferred to Hybond N (Amersham) membranes using a slot blot apparatus. One hundred microlitres was added to each slot, and then washed through with 10x SSC. RNA was fixed by exposing to UV light for 5 minutes at 305 nm, the filter was allowed to dry.

The membrane was prehybridised overnight at 37° C in 50% (v/v) formamide, 4x phosphate buffered saline sodium citrate (SSCP), 1x Denharts and 0.1% SDS (w/v) in a volume of 100ml. Radiolabelled cDNA probe was prepared as described (Section 2.8.a.). Twenty millilitres of hybridisation buffer, 50% formamide, 4x SSCP, 1x Denharts was prepared on ice. Probe and sufficient sheared calf thymus to give a 50ug/ml final concentration were boiled for 10 minutes, rapidly chilled on ice and added to hybridisation buffer. The membrane was incubated for 20 hours at 37° C in hybridisation mix, and then washed in 2x SSC/0.1% SDS, 1x SSC/0.1% SDS, 0.5x SSC/0.1% SDS, and 0.1x SSC/0.1% SDS. Membranes were autoradiographed for 3-4 days at -70° C with Hyperfilm-MP (RP N6, Amersham), using intensifying screens.

To reprobe a filter the membrane was first stripped by incubating at 65° C for 1-2 hours in 0.005M Tris-HCl at pH 8.0 with 0.002M Na_2EDTA and 0.1x Denharts. Autoradiography was used to confirm that stripping was effective.

2.9. ANALYSIS OF PROTEINS

2.9.a. Western Blotting

Protein samples were analysed on 10% or 15% SDS-PAGE gels according to the method of Laemmli (1970). A discontinuous system that stacks at pH 6.8 and resolves at pH 8.8 was used. Samples were treated by adding an equal volume of 2x loading dye (Section 9.5.), if run under reducing conditions 100mM DTT or 1% 2-mercaptoethanol were added before boiling for 10 minutes. Ten microlitres of rainbow markers/well (Molecular weight 14.3-200 kDa; Amersham) were treated in the same way. Gels were run at 25mA in an SDS-Tris glycine buffer, and were then transferred onto nitrocellulose membranes (Bio-Rad, Hertfordshire) at 220mA for two hours.

2.9.b. Protein detection

Radiolabelled samples were detected directly from gels using autoradiographic techniques. Preparations containing ^{125}I-label were wrapped and directly autoradiographed.

2.9.c. Immunochemical IL-1 beta detection

Interleukin 1 beta in samples blotted onto nitrocellulose filters was analysed using two immunochemical detection schemes; the first was used when recombinant pro IL-1 beta was the substrate of reactions, the second, a more sensitive avidin-biotin system was used when THP-1 cell lysates served as a source of the IL-1 precursor. Solutions used are described in Section 9.5..

In the former method filters were first blocked in a 5% (w/v) BSA (RIA grade; Sigma) solution for an hour at room temperature. This was followed by an overnight incubation in sheep anti-IL-1 beta antiserum at 1:2000 dilution (Glaxo Group Research), in blocking buffer. Blots were washed three times, for five minutes each wash, in a 20mM Tris/0.15M NaCl solution with 0.05% Tween 20, followed by a detergent free wash. Second antibody, alkaline phosphatase conjugated anti-sheep IgG (Sigma), was diluted 1:2000 in second antibody buffer and filters incubated for 4 hours. This was followed by further washing. Both substrates were prepared at a concentration of 20mg/ml

immediately before use in substrate buffer: Fast Blue RR was obtained as a crystalline salt (Sigma, F 0500) and Napthol AS-MX phosphate as the sodium salt (Sigma, N 5000). Solutions were mixed and added to filters for up to 15 minutes, the reaction was stopped by washing blots several times in PBS (Section 9.3.a.).

The alternative method followed the Vector (Peterborough) kit protocol issued with reagents. Filters were blocked for 30 minutes at room temperature in a Tris/NaCl/Tween 20 solution (TTBS), this was also used for antibody dilution and washing. Primary antibody, sheep anti-IL-1 beta antiserum (NIBSC), was incubated in the same manner at a 1:500 dilution, this was followed by three 5 minute washes in TTBS. Biotinylated rabbit anti-sheep antibody (Vector) was added at a 5ug/ml concentration. The avidin-biotin alkaline phosphatase complex (Standard ABC-AP kit, Vector) was prepared 30 minutes before use, by adding 100ul of reagents A and B to 20ml of TTBS. Three drops each of substrate solution A, B, and C (Vector Red, Kit 1) were added to 20ml of 0.1M Tris-HCl at pH 8.2, this was mixed and added to the washed filters until bands had developed. The reaction was stopped by washing in distilled water.

3. RESULTS: Plasma interleukin 1 measurement

3.1. INTRODUCTION

The measurement of IL-1 in biological fluids is known to be subject to inaccuracies due to the presence of specific and non-specific cytotoxic and inhibitory factors that affect bioassays. Other growth factors eg IL-2, IL-4, IL-6 can also give false positive readings. Much of the data concerning *ex vivo* IL-1 measurement has been obtained from fluids after partial purification, usually by gel filtration.

In synovial fluid from rheumatoid arthritis patients, inhibitory activity in the LAF assay can be removed by gel filtration which allows the detection of an IL-1-like activity (Miossec *et al*, 1986). Recent results show both IL-1 forms in synovial fluid as measured by immunoassay, and demonstrate inhibitory activity on D10 murine T cells at dilutions less than 1:1000 (di Giovine *et al*, 1990). Tamatani *et al* (1988) used gel filtration to characterise IL-1 bioactivities in amniotic fluid. Attempts to measure IL-1 in urine have shown inhibitory effects in the majority of samples (Kimball *et al*, 1984), probably due to the subsequently well characterised IL-1 inhibitor (Balavoine *et al*, 1986).

In serum IL-1 has been demonstrated by a D10 bioassay, thymocyte proliferation and LEM assay (Cannon and Kluger, 1983; Cannon and Dinarello, 1985), all require fractionation before assay. Gahring *et al* (1984) identified IL-1 in a low molecular weight fraction of plasma (<25 kDa), this activity could be completely inhibited if reconstituted with the high molecular weight fraction. Serum IL-1 suppressor activity has been demonstrated at a size of 50 kDa or larger, this can be separated to reveal IL-1 bioactivity even in samples from normal individuals (Luger *et al*, 1986; Cannon and Dinarello, 1984).

More confident measurement of blood IL-1 levels can now be made due to the commercial availability of immunoassays specific for IL-1 alpha and beta. However there is still the possibility that high protein and lipid levels in plasma and serum samples may interfere with IL-1 detection in some assays. This may be further complicated in disease states. A recent report describes an extraction protocol designed to overcome these problems, by means of a

chloroform extraction step (Cannon *et al*, 1988).

The accurate determination of circulating IL-1 levels is important to the understanding of the *in vivo* role of IL-1 in both normal and disease states. The present study describes the measurement of plasma IL-1 alpha and beta by immunoassay, and the effect of extraction on detection systems. The concentrations of circulating IL-1 in normal individuals and rheumatoid arthritis patients were also compared.

3.2. PLASMA INTERLEUKIN 1 BETA MEASUREMENT

3.2.a. Assay validation

3.2.a.i. Interleukin 1 beta recovery

Control and rheumatoid arthritis patient plasma samples were prepared as described in Section 2.3.a.. They were then spiked with 200pg/ml IL-1 beta standard either before or after chloroform extraction (Section 2.3.d.), recovery of added IL-1 was determined by measurement in the Cistron IL-1 beta ELISA (Section 2.4.a.i.). The percentage recovery of added IL-1 is shown in Table 3.2.1., mean values were above 80% for both populations irrespective of when the standard was added.

3.2.a.ii. Dilution characteristics

Plasma samples were serially diluted in the ELISA buffer before assay. Dilution curves are shown in Figure 3.2.1., the samples showed parallel dilution characteristics.

3.2.a.iii. Interassay variability

Duplicate samples were tested for IL-1 beta levels in separate Cistron ELISA kits, and the mean plasma concentrations were compared. As shown in Table 3.2.2. the difference between assays was less than 12%.

3.2.a.iv. Plasma effects on IL-1 detection

Two comparable IL-1 beta standard curves were prepared. From the stock

Sample	Recovery % A	B
Patient		
1	97	127
2	85	104
3	71	-
4	81	88
5	81	98
6	87	-
7	68	89
8	91	105
9	83	71
10	78	101
Mean±SEM	82.2±2.7	97.9±2.8
Controls		
1	69	68
2	71	101
3	120	73
4	127	130
Mean±SEM	96.8±15.2	94.0±15.2

Table 3.2.1. Recovery of added IL-1 beta from plasma

A = % recovery of 200pg/ml IL-1 beta added to plasma before chloroform extraction.

B = % recovery of 200pg/ml IL-1 beta added to plasma following chloroform extraction.

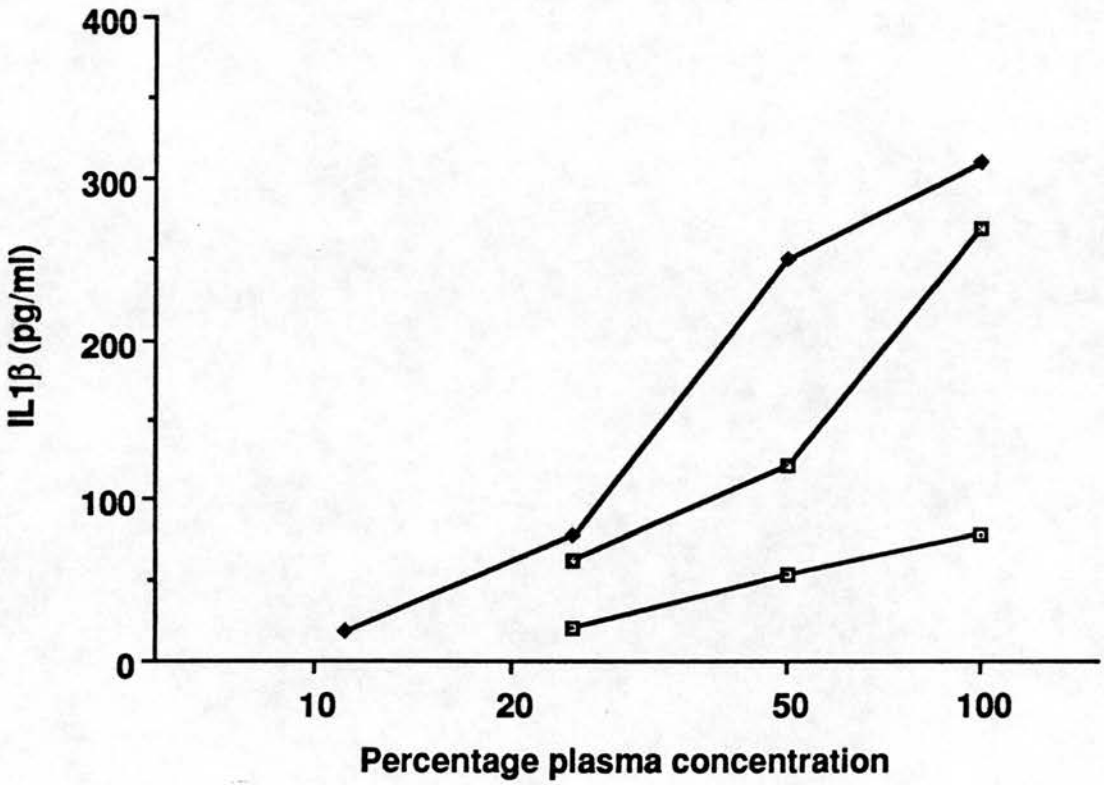

Figure 3.2.1. Dilution characteristics of plasma IL-1 beta

Three plasma samples were serially diluted in buffer before assay in IL-1 beta ELISA. Dilutions are expressed as percentage plasma (v/v) in the ELISA.

Sample	IL-1 beta pg/ml Assay A	Assay B
1	<20	50
2	130	110
3	70	65
4	120	125
5	54	88
6	165	160
7	<20	<20
8	56	100
Mean	79.4	89.5
Variability	11.5%	

Table 3.2.2. IL-1 beta ELISA assay variability

Time hr	IL-1 beta pg/ml -LPS	+LPS
0	115	115
0.5	149	175
1.0	29	23
1.5	40	82
2.0	47	35
3.0	52	100

Table 3.2.3. Sample collection validation

IL-1 preparation dilutions were made in either the Cistron BSA-phosphate buffer or in a plasma sample known to contain little endogenous IL-1 beta (<20pg/ml). The resultant standard curves (Figure 3.2.2.) showed similar profiles but plasma appeared to reduce the level of detection at higher concentrations.

3.2.a.v. Effect of rheumatoid factor

To ensure that rheumatoid factor was not influencing the assay, the mean plasma IL-1 beta concentrations were compared with rheumatoid factor titre in the patient group. Those patients with detectable rheumatoid factor had slightly higher mean IL-1 levels (162pg/ml compared with 130pg/ml), but no correlation was seen between antibody titre and IL-1 beta concentration (Figure 3.2.3.).

3.2.a.vi. Validation of sample collection

The effect of delayed sample preparation was assessed by allowing blood samples to stand at 4° C for up to three hours either with or without addition of 100pg/ml of LPS (Section 2.3.a.). The results are shown in Table 3.2.3.. Samples left for more than thirty minutes lost considerable IL-1 beta immunoreactivity (from an initial concentration of 115pg/ml to a mean value of 26pg/ml after 1 hour). However the presence of LPS in the collection tube led to a rapid increase in levels at between 2-3 hours (from 35pg/ml at 2 hours to 100pg/ml after 3 hours incubation).

3.2.b. Effect of plasma extraction

Eleven control and 35 RA patient plasma samples were measured in the IL-1 beta assay both before and after chloroform extraction. The mean values for each group were compared using Students T-test. As shown in Table 3.2.4. extraction significantly increased the amount of detectable IL-1 beta in both groups. In the control group detectable IL-1 beta levels rose from a mean of 20.3pg/ml to 33.4pg/ml following extraction. The assay sensitivity limit was 20pg/ml, so negative samples were allocated a value of 19pg/ml to calculate a

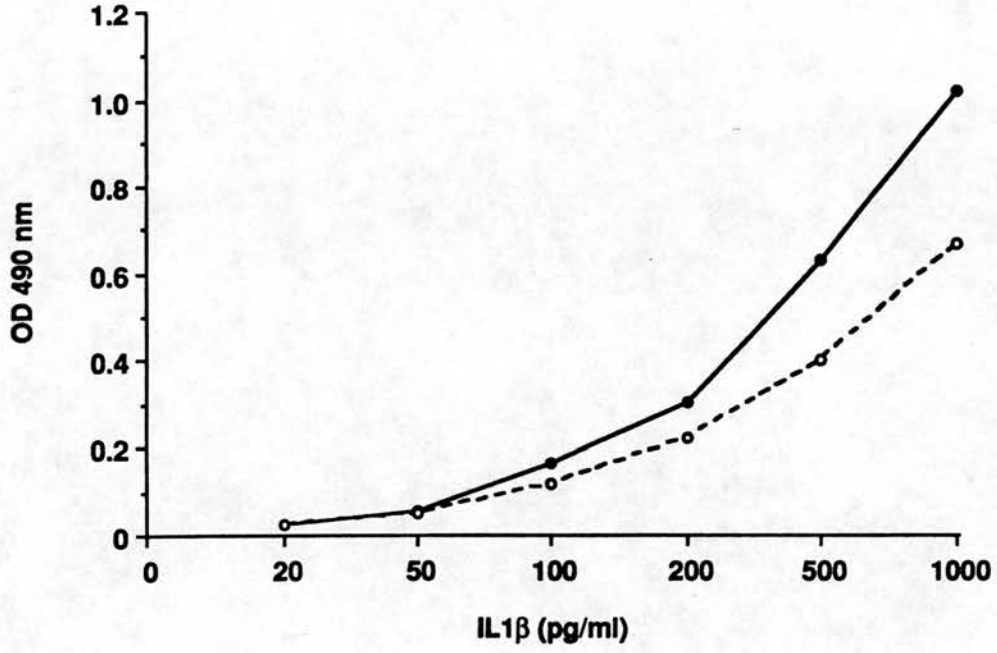

Figure 3.2.2. The effect of plasma on the IL-1 beta ELISA standard curve
The IL-1 beta standard curve was prepared in both assay buffer (——) and a plasma sample (- - -) containing low endogenous IL-1 beta levels.

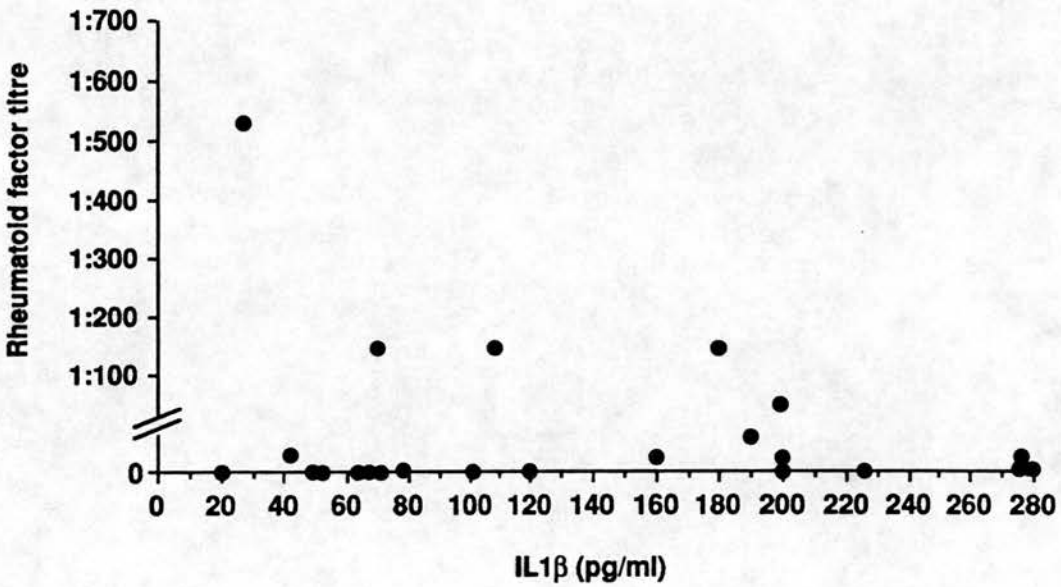

Figure 3.2.3. Correlation of plasma IL-1 beta levels with rheumatoid factor titre

Rheumatoid factor titre in patients was correlated with plasma IL-1 beta concentration. No significant correlation was seen (n= 23, r= 0.25, $p<0.1$).

	Control	RA
n	11	35
IL-1 beta pg/ml		
Unextracted	20.3	54.5
Extracted	33.4	85.6
	p<0.001	p<0.05

Table 3.2.4. Effect of extraction on IL-1 beta detection in plasma

	Plasma IL-1 beta pg/ml	
	Controls	RA
n	21	51
Range	20-78	20-230
Median	50	90
Mean	44.7	98.2*
SEM	4.4	7.9

*Control *vs* RA t=4.28; p<0.0001

Table 3.2.5. Interleukin 1 beta in extracted plasma

group mean. RA patients showed a rise in plasma IL-1 beta from 54.4pg/ml to 85.6pg/ml after chloroform treatment.

3.2.c. Plasma IL-1 beta levels

3.2.c.i. Comparison of control and RA groups

The mean plasma IL-1 beta concentration following extraction was compared for patients and controls. The group of 21 normals showed significantly lower mean IL-1 beta values (44.7pg/ml) than 51 age matched rheumatoid patients (98.2pg/ml), as assessed by Students T-test (Table 3.2.5.).

3.2.c.ii. IL-1 beta correlation with disease activity

The correlations between plasma IL-1 beta levels and various measures of disease activity were determined. Figure 3.2.4. shows a significant cross-sectional correlation between Ritchie articular index and plasma IL-1 beta, in an RA population of 37 ($r= 0.572$, $p<0.001$). Cross-sectional correlations were also seen with erythrocyte sedimentation rate ($r= 0.358$, $p<0.05$; Figure 3.2.5.), duration of morning stiffness ($r= 0.473$, $p<0.005$) and pain score ($r= 0.605$, $p<0.001$). A negative correlation with haemoglobin concentration was seen ($r= -0.53$, $p<0.001$; Figure 3.2.6.). Significance was not reached in correlations with platelet count or white cell count.

3.2.c.iii. Serial IL-1 beta measurement

Serial blood samples were taken from hospitalised RA patients and longitudinal IL-1 beta measurements compared with disease assessment. Figure 3.2.7. shows three individuals with their plasma IL-1 beta levels correlated against Ritchie score, platelet count, and ESR. In each case the patient was admitted with active disease that subsided over the first few days. This was subsequently followed in each of these cases by an increase in disease activity. Patient A shows correlations between IL-1 beta and ESR ($r= 0.74$, $p<0.02$), and platelet count ($r= 0.745$, $p<0.02$). In patient C there are significant correlations, between plasma IL-1 and; platelet count ($r= 0.855$,

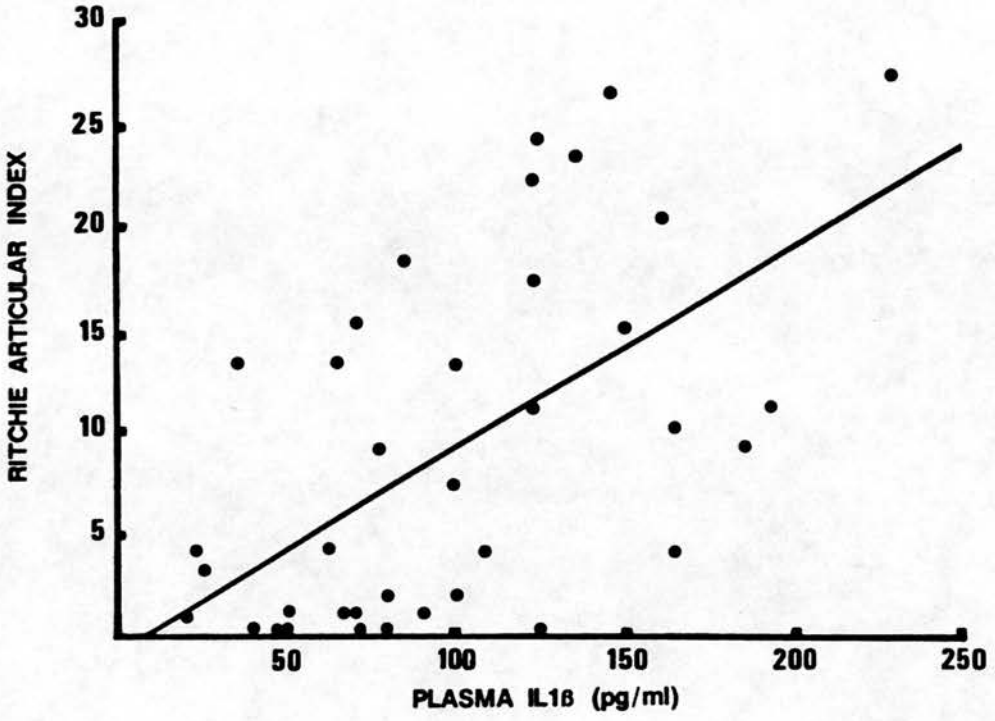

Figure 3.2.4. Correlation between plasma IL-1 beta and Ritchie articular index

Plasma IL-1 beta concentration was correlated with Ritchie articular score in a group of RA patients. A significant correlation was seen, n= 37, r= 0.572, $p<0.001$.

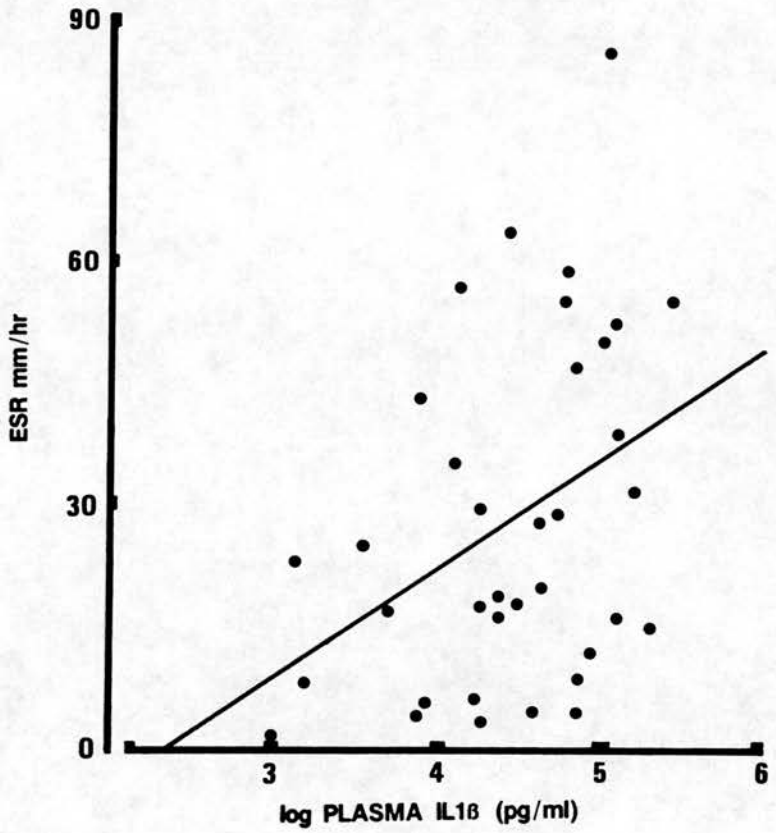

Figure 3.2.5. Correlation between plasma IL-1 beta and ESR

Plasma IL-1 beta level was compared with ESR, as a laboratory measure of disease activity. IL-1 beta results were plotted on a natural log scale. A significant correlation was seen n= 35, r= 0.358, $p<0.05$.

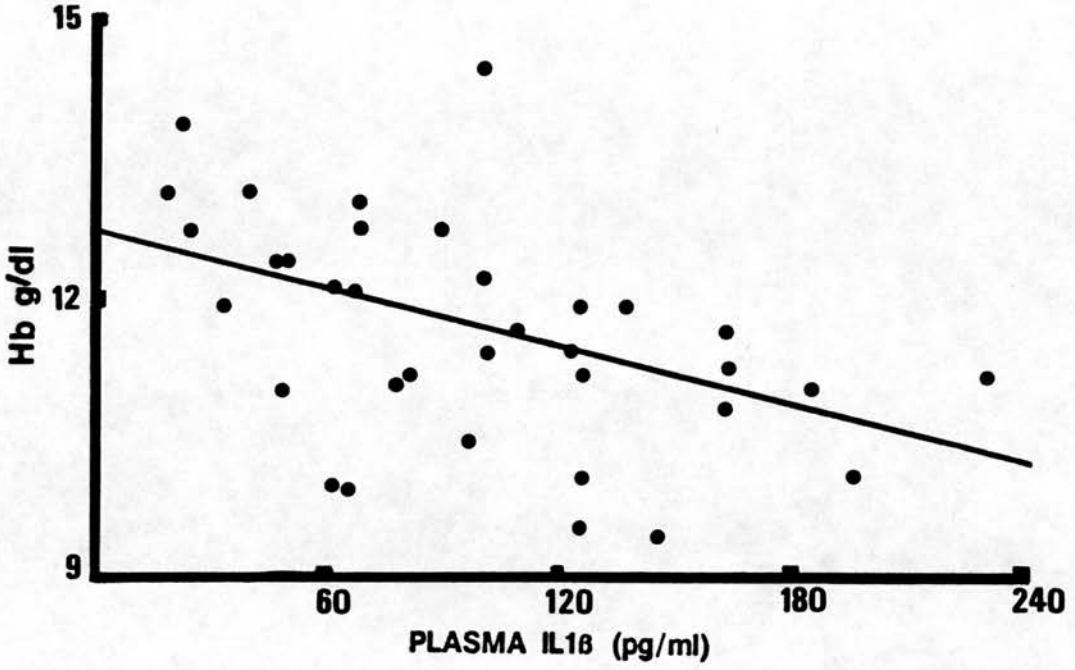

Figure 3.2.6. Negative correlation between plasma IL-1 beta and haemoglobin concentration

Blood haemoglobin concentration showed a significant negative correlation with plasma IL-1 beta levels in the patient group (n= 36, r= -0.53, $p<0.001$).

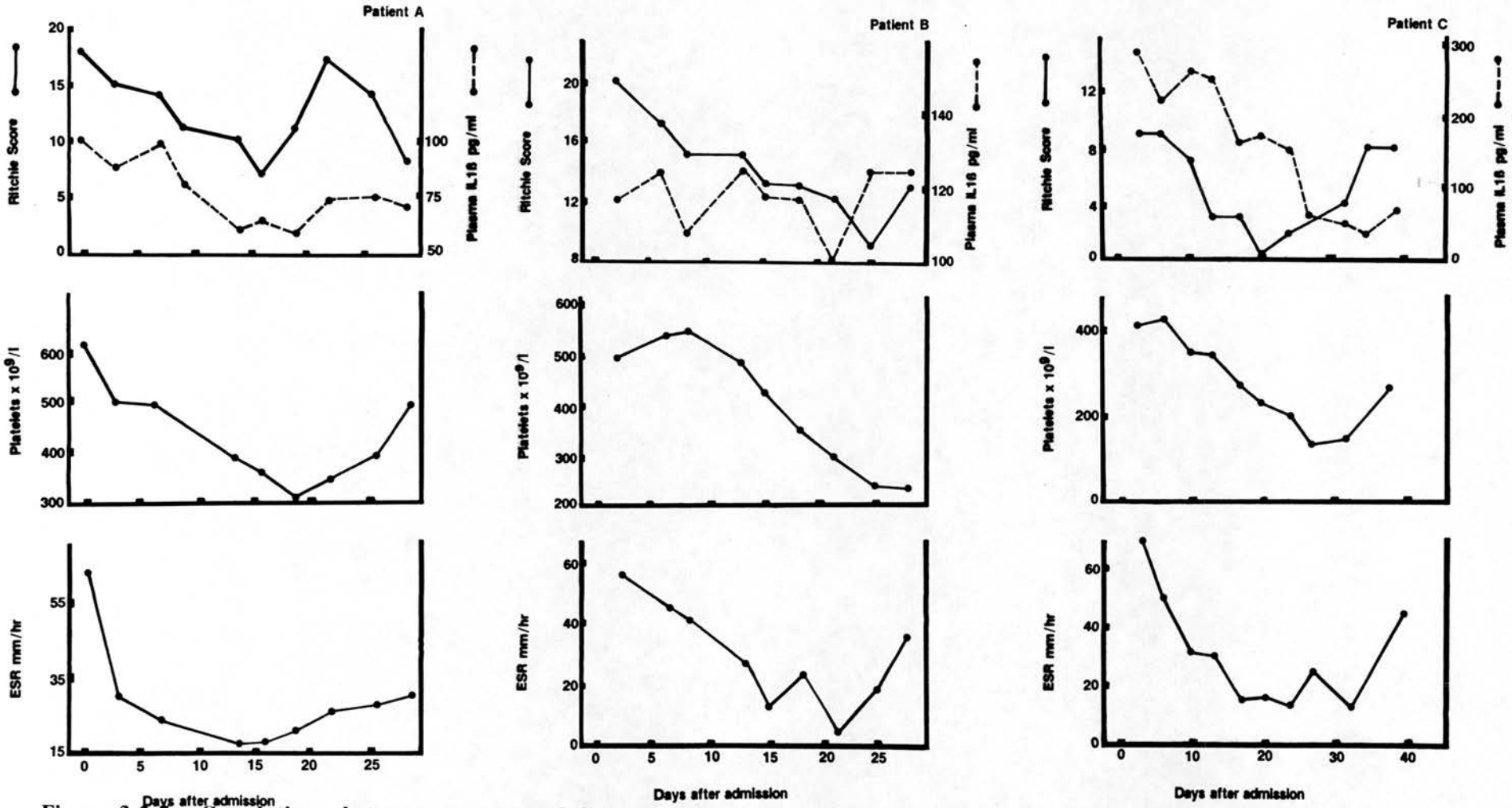

Figure 3.2.7. Correlations between measures of disease activity and plasma IL-1 beta in patients tested longitudinally

Plasma IL-1 beta levels and activity scores in patients admitted to hospital with RA are shown. Plasma IL-1 beta (upper panel, ---) correlated with Ritchie index (upper panel, ——), platelet count (middle panel) and ESR (lower panel). Patient A showed correlations with ESR (n= 10, r= 0.74, p<0.02) and platelet count (n= 10, r= 0.745, p<0.02), no significant values were found with Patient B. Patient C showed correlations with ESR (n= 10, r= 0.618, p<0.05), platelet count (r= 0.855, p<0.005) pain score (r= 0.794, p<0.005), and white cell count (r= 0.806, p<0.005).

$p<0.005$), white cell count ($r= 0.806$, $p<0.005$), pain score ($r= 0.794$, $p<0.01$) and ESR ($r= 0.618$, $p<0.05$). Patient B showed similar trends but no significant correlations.

3.2.d. Comparison of IL-1 beta immunoassays

To assess the validity of the Cistron ELISA, extracted plasma IL-1 beta measurements were compared with those values obtained for the same samples in a Roussel ELISA (described in Section 2.4.a.ii.). The Cistron ELISA read slightly higher (mean levels 96.5pg/ml and 70.9pg/ml) but correlated with the Roussel assay results in the majority of samples ($n= 42$, $r= 0.711$, $p<0.001$ (Figure 3.2.8.). However several samples gave very high readings in the Roussel kit (350-2000pg/ml) but comparatively low ones in the Cistron ELISA (20-258pg/ml). The reason for this was unclear.

3.3. PLASMA INTERLEUKIN 1 ALPHA MEASUREMENT

3.3.a. Assay validation

3.3.a.i. IL-1 alpha recovery

Plasma was extracted and 200pg/ml of standard IL-1 alpha was added. The recovery was tested in an Amersham radio-immunoassay (Section 2.4.b.). The results are shown in Table 3.3.1.. The mean level of recovery of exogenous IL-1 alpha was 88.1±7.5%.

3.3.a.ii. Dilution characteristics

Plasma samples were diluted serially in assay buffer before RIA. The dilution characteristics are shown in Figure 3.3.1., linear dilution of endogenous IL-1 alpha was seen.

3.3.a.iii. Assay variability

Duplicate plasma samples were tested for immunoreactive IL-1 alpha levels in different assays, the mean values from each were compared. As shown in Table 3.3.2. the interassay variability was 9.8%.

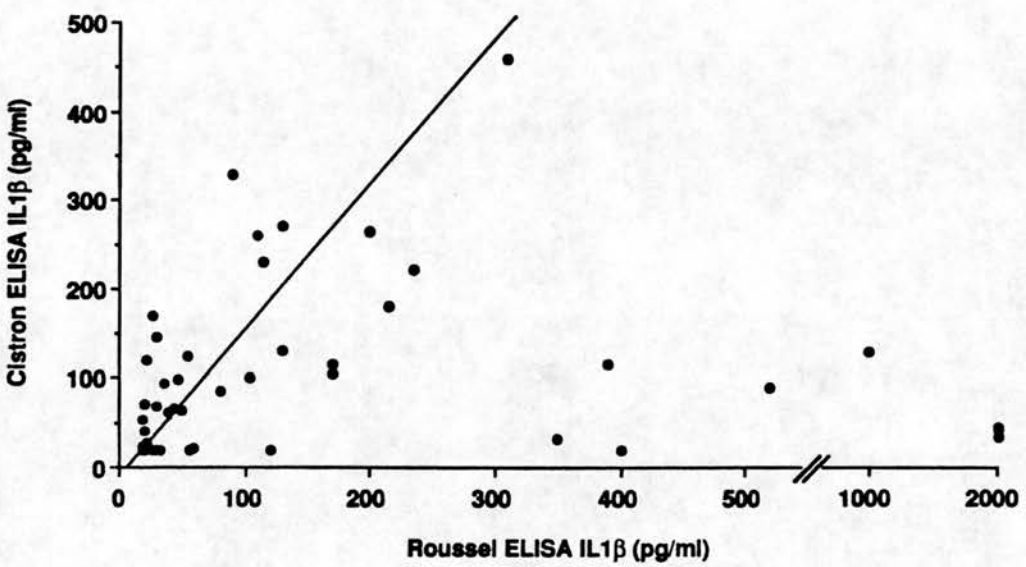

Figure 3.2.8. Comparison of plasma IL-1 beta detection by two ELISAs

Plasma IL-1 beta levels were measured, following extraction, in Cistron and Roussel ELISAs. All the plasma samples tested are shown. A selected population was compared for correlation between ELISAs. The population included all samples that gave a value of less than 325 pg/ml in the Roussel assay. A significant correlation was seen n= 41, r= 0.711, $p<0.001$.

Sample	Recovery %
1	94
2	105
3	108
4	62.5
5	98
6	92
7	57.7
Mean	88.1%

Table 3.3.1. Recovery of added IL-1 alpha

Sample	IL-1 alpha pg/ml Assay 1	Assay 2
1	440	312
2	530	593
3	170	110
4	155	175
5	230	200
6	128	360
7	102	200
8	205	230
9	300	300
Mean	251	275.6
Difference	9.8%	

Table 3.3.2. Variability of IL-1 alpha RIA

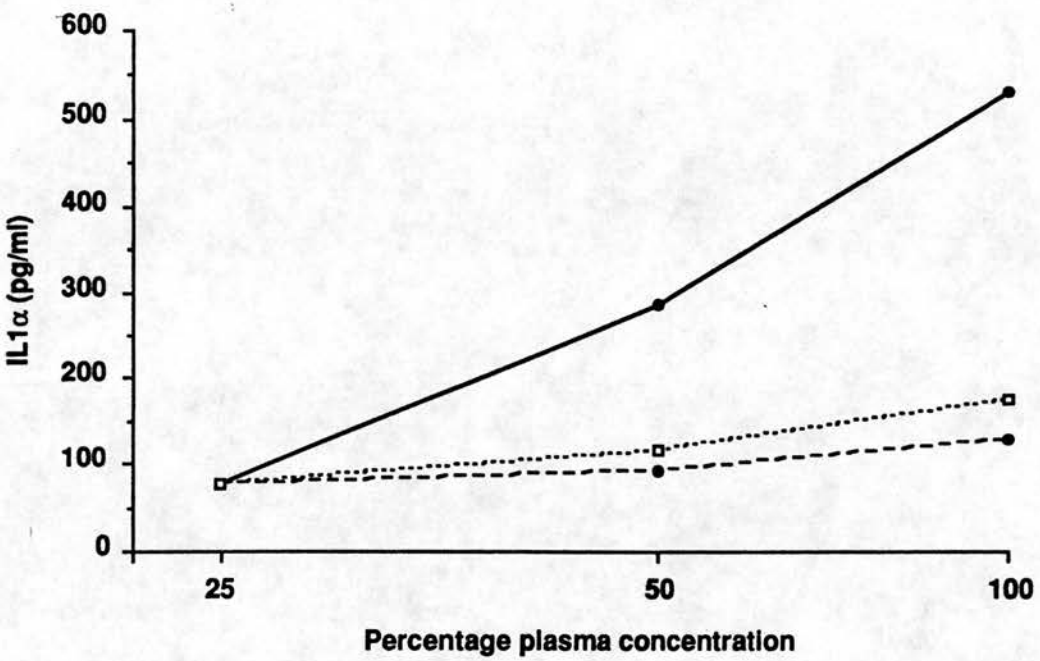

Figure 3.3.1. Dilution characteristics of plasma IL-1 alpha

Plasma samples were serially diluted in assay buffer before being analysed by IL-1 alpha RIA. Sample dilutions are expressed as percentage (v/v) plasma.

3.3.a.iv. Validation of sample collection

Blood samples were allowed to stand for up to three hours at 4° C, with or without added LPS (100pg/ml) as described in Section 2.3.a.. Plasma was prepared, extracted and tested for IL-1 alpha immunoreactivity. The results are shown in Table 3.3.3.. The initial level of IL-1 alpha dropped during the first hour (from a mean of 95pg/ml to 80pg/ml) and then rose to considerably higher concentrations after 1.5 hours. Addition of LPS resulted in a greater peak IL-1 alpha level at this time point (125pg/ml without and 200pg/ml with LPS). In both cases by the third hour IL-1 immunoreactivity had fallen to around the detection limit of the assay.

3.3.b. Effect of plasma extraction

Plasma samples from both control and RA patient groups were tested in the IL-1 alpha RIA before and after extraction. The results are shown in Table 3.3.4.. A group of 17 normal individuals showed a non-significant increase in plasma IL-1 alpha after extraction (from 222pg/ml to 249pg/ml). The group of 44 patients, however, showed a slight but significant increase in IL-1 alpha levels (from 192pg/ml to 238pg/ml).

3.3.c. Plasma IL-1 alpha levels

3.3.c.i. Comparison of control and RA groups

The mean plasma IL-1 alpha levels were compared between age matched patient and control groups (Table 3.3.5.), similar concentrations were seen in the two groups (control mean 239pg/ml, patient mean 245pg/ml). There was no significant difference in plasma IL-1 alpha concentrations as determined using Students T-test.

3.3.c.ii. IL-1 alpha correlation with disease activity

The plasma IL-1 alpha concentration was compared with various measures of disease activity within the patient group. In contrast to IL-1 beta no correlation was seen with clinical or laboratory measures of disease activity, apart from a correlation reaching slight significance with ESR, the

Time hr	Plasma IL-1 alpha pg/ml -LPS	+LPS
0	80	110
0.5	118	135
1.0	82	<78
1.5	125	200
2.0	120	180
3.0	<78	80

Table 3.3.3. Validation of sample collection

	Control	RA
n	17	44
IL-1 alpha pg/ml		
Unextracted	222.7	192.5
Extracted	249.9	238.5
	p<0.08	p<0.0003

Table 3.3.4. Effect of extraction on plasma IL-1 alpha detection

	Plasma IL1 alpha (pg/ml)	
	Control	RA
n	33	53
Range	110-620	110-500
Median	220.0	230.0
Mean	239.5	245.5
SEM	14.7	10.5

Controls vs RA, t=0.338, p=0.736

Table 3.3.5. Interleukin 1 alpha levels in extracted plasma

results (n= 48, r= 0.29, $p<0.05$) are shown in Figure 3.3.2..

3.3.c.iii. Serial IL-1 alpha measurement

Serial plasma samples were taken from hospitalised RA patients, and plasma IL-1 alpha measurements compared to conventional measures of disease activity. Results from three patients are shown in Figure 3.3.3.. In Patient A correlations were found with Ritchie joint index (r= 0.678, $p<0.005$), pain score (r= 0.59, $p<0.02$), duration of morning stiffness (r= 0.538, $p<0.5$) and haemoglobin concentration (r=-0.49, $p<0.05$). Patient B showed significant correlations with duration of morning stiffness (r= 0.587, $p<0.02$) and platelet count (r= 0.719, $p<0.005$). In Patient C similar trends were observed, with decreased IL-1 alpha levels corresponding to clinical improvement, though correlations did not reach statistical significance.

3.4. DISCUSSION

The results show that chloroform extraction significantly increased the amount of detectable plasma IL-1 beta immunoreactivity in both normal and RA groups. The mean concentration of plasma IL-1 beta in the patient population was significantly higher than that in controls. Furthermore plasma IL-1 beta also correlated with various measures of disease activity, in both the whole patient population and in individuals tested at regular intervals during a period of hospitalisation. Use of an alternative IL-1 beta ELISA showed that the assays were comparable for the majority of samples, but that the second ELISA gave unexpectedly high readings in some cases. These levels are considerably higher than those seen using other assays in our group.

In contrast, plasma IL-1 alpha levels in most individuals were unaffected by extraction, and no difference was seen in the mean concentrations between patient and control groups. The patient population as a whole showed little correlation between IL-1 alpha and indices of disease activity. However, examination of serial samples from particular individuals resulted in significant correlation of plasma IL-1 alpha levels with clinical

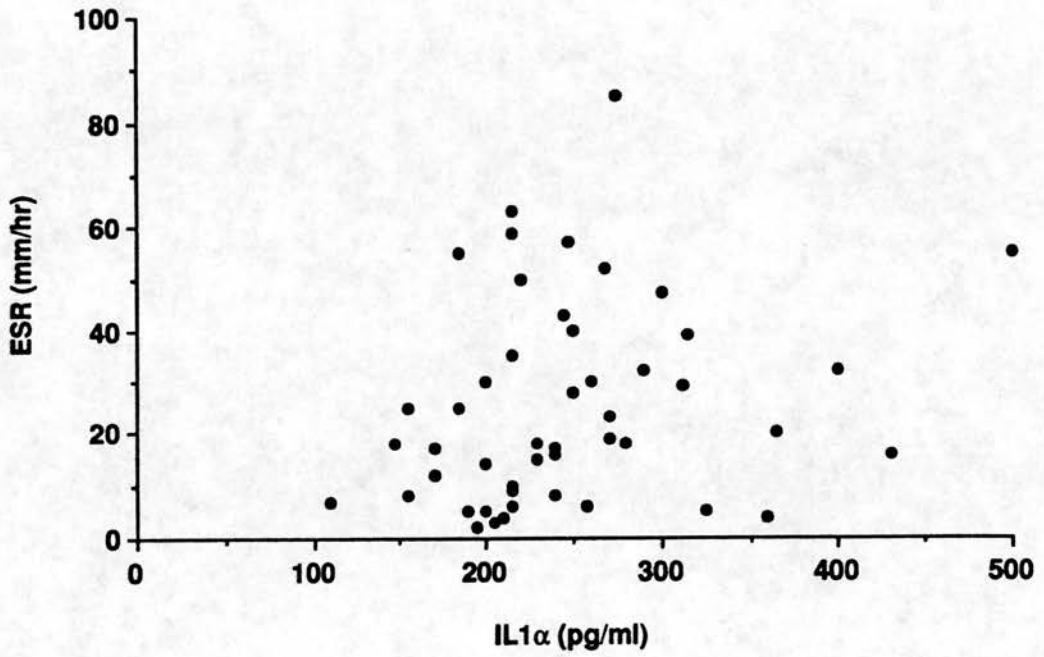

Figure 3.3.2. Comparison of plasma IL-1 alpha levels with ESR in RA patients
Extracted plasma samples were tested for IL-1 alpha level and the values compared with blood ESR measurement. A slight correlation was seen (n= 48, r= 0.29, $p<0.05$).

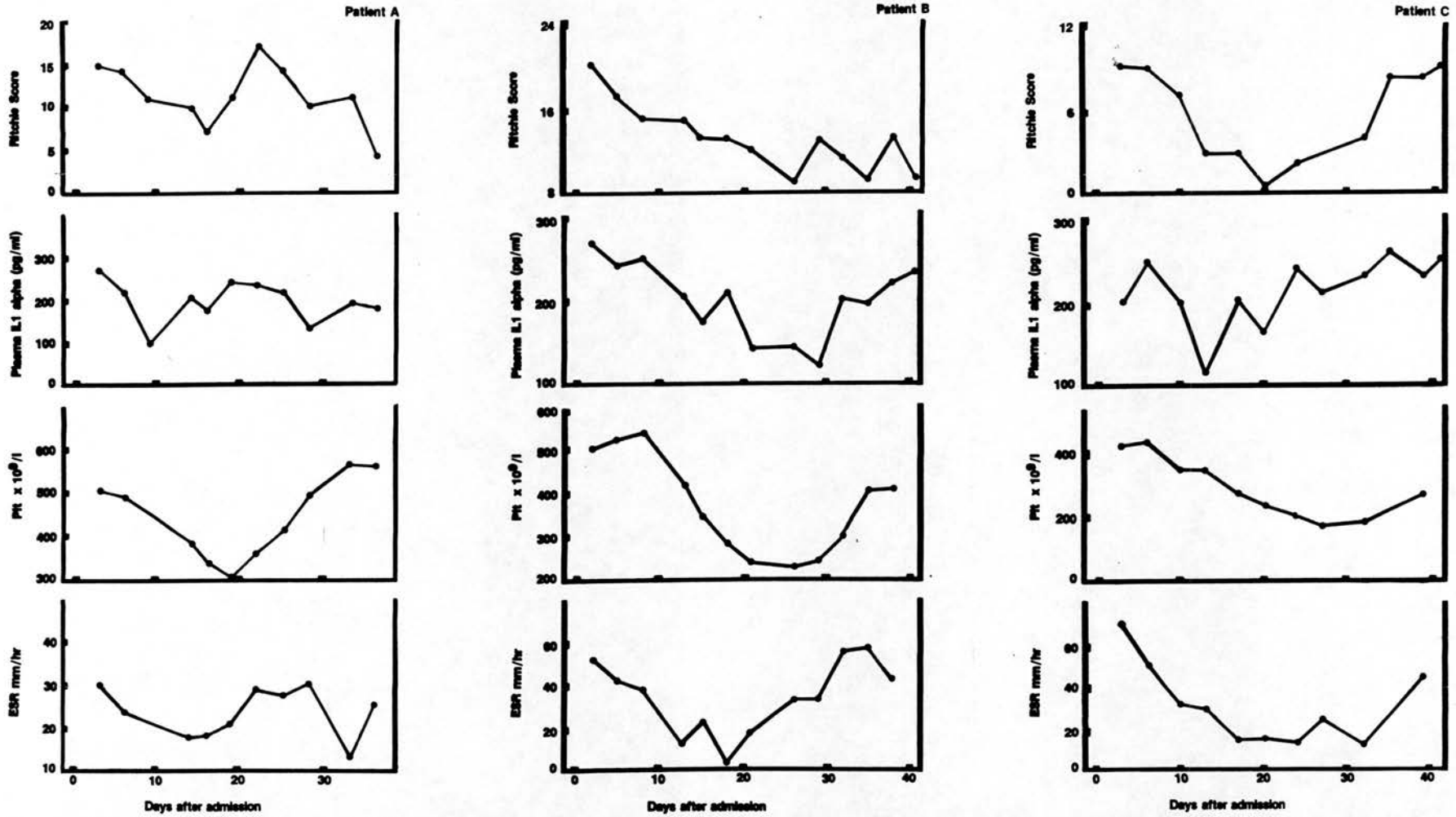

Figure 3.3.3. Correlation between measures of disease activity and plasma IL-1 alpha in patients tested longitudinally

Plasma IL-1 alpha levels, Ritchie index, platelet count and ESR in patients admitted to hospital with RA are shown. Patient A showed correlations with Ritchie index (n= 10, r= 0.678, $p<0.005$), pain score (r= 0.59, $p<0.02$), duration of morning stiffness (r= 0.538, $p<0.05$) and a negative value with haemoglobin concentration (r= -0.49, $p<0.05$). Patient B showed correlations with duration of morning stiffness (n= 13, r= 0.587, $p<0.02$) and platelet count (n= 12, r= 0.719, $p<0.005$). Patient C though exhibiting similar trends did not show significant correlations.

and laboratory disease assessments.

Examination of blood collection methods showed that both IL-1 alpha and beta rapidly degraded if plasma was not prepared soon after collection and LPS contamination could result in raised levels after longer time periods. The importance of careful blood sampling techniques was emphasised by Cannon *et al* (1988), who suggested that plasma samples give more accurate representations of the *in vivo* levels than serum samples, as IL-1 may be released during the clotting process. The importance of including protease inhibitors to prevent degradation of IL-1 has also been discussed. The combination of aprotinin and EDTA has been previously described (Dinarello *et al*, 1984). However, even when protease inhibitors are included during blood collection a loss of immunoreactive IL-1 is observed if samples are not prepared rapidly. If allowed to stand for longer than two hours even at 4° C IL-1 may be released from cells, this may be further complicated by the presence of LPS.

Chloroform extraction was seen to be important in unmasking IL-1 beta immunoreactivity in both patient and control groups. The initial description of this protocol, using a specific IL-1 beta RIA, showed that extraction removed the influence of non-specific factors that interfere with immunoassay in the plasma of malarial patients (Cannon *et al*, 1988). The latter report described comparable IL-1 beta levels in normal individuals (mean= 62pg/ml) to those reported here.

Using other assays it has been reported that chloroform extraction of plasma samples does not improve detection of IL-1 beta (Fontaine *et al*, 1989). We therefore decided to compare IL-1 levels in extracted plasma as measured by the Roussel and Cistron ELISAs. The majority of samples showed comparable IL-1 beta levels in both assays, indicating that while extraction may not be absolutely necessary, it was certainly not detrimental to detection in the Roussel ELISA. This assay did however, show unexpectedly high levels in some samples (>2000 pg/ml). Plasma beta IL-1 concentrations at these levels have not been reported in other systems. It is reasonable to assume that these extremely high levels are artefactual. Other commercially

available immunoassays are unable to measure IL-1 beta in extracted or unextracted plasma (J A Symons personal communication). This suggests that plasma IL-1 immunoreactivity may be masked at certain epitopes and that extraction uncovers some but not all of these sites. Thus the success of immunodetection depends upon the particular epitopes of IL-1 that are recognised by the antibodies employed and an ideal system can only be designed when the epitope structure of natural IL-1 is fully resolved. In contrast to IL-1 beta, IL-1 alpha detection is not increased by chloroform extraction in the majority of samples.

Other extraction protocols have been described including a silica elution method (Series *et al*, 1988). This however, results in only 30% recovery of IL-1 added to blood, and detection of levels in the range of 0.3-0.6 pg/ml.

The measurement of IL-1 in plasma from rheumatoid arthritis patients shows that their mean plasma IL-1 beta level is raised compared to a normal population. It is known that during active RA IL-1 immunoreactivity is found in the joint fluid (Symons *et al*, 1989) and that the mRNA can be identified in the synovial lining (Duff *et al*, 1988). It may be, therefore, that plasma IL-1 beta is seen as a result of a local inflammatory activation within the joint. The correlation between IL-1 beta concentration and laboratory measures of disease activity, such as ESR, indicate a role for the former in the activation of the acute phase response. Interleukin 1 is also known to have effects on both iron metabolism (Dinarello, 1984) and on the a suppressive action on the development of erythroid progenitor cells (Maury *et al*, 1988). The negative correlation between IL-1 and blood haemoglobin concentration provides further evidence that the cytokine may play a part in the refractory anaemia often seen in chronic inflammatory disease. An involvement for IL-1 in the RA disease process has often been suggested when considering its ability to activate synovial cells, cause resorption of bone and cartilage and act as a chemoattractant for cells involved in the inflammatory response. Therefore, evidence of raised plasma IL-1 levels in

active stages of the disease lends considerable support to this idea.

It has been suggested that individuals can be divided into high and low producers of IL-1 (Molvig *et al*, 1988). It is suggested that these inter-individual differences are related to HLA haplotype (Santamaria *et al*, 1989). This difference was also observed at the plasma IL-1 beta level, the control population having low levels presumably due to minimal cellular activation, whereas serial measurements from some patients eg. Patient A, showed variations within a small range of IL-1 beta concentrations but others eg. Patient C showed a high levels of production. This variation in the IL-1 beta levels produced by particular individuals may explain the scatter seen in IL-1 population data. In contrast IL-1 alpha remained at comparable levels in individuals tested serially.

Immunoassay of IL-1 may detect biologically inactive IL-1 in the form of: pro IL-1 beta, partial degradation products and complexes of IL-1 with other proteins. The *in vivo* effects of inhibitors can also not be determined by immunoassay. Recent evidence suggests that extracellular precursor IL-1 molecules are likely to be processed rapidly *in vivo* by proteolytic enzymes produced, particularly at an inflammatory site (Hazuda *et al*, 1990).

The correlations found in the RA population suggest that immunoreactive blood IL-1 beta levels do, indeed, reflect production of bioactive IL-1, within the joint.

Plasma interleukin 1 alpha levels showed no difference between control and patient groups, and no population correlations with measures of disease activity. Individuals undergoing serial measurement did however show correlations between plasma IL-1 alpha and assessment of disease activity. It is therefore suggested that the basal level of IL-1 alpha production is high and may be derived from an organ such as the skin. It has been shown that human keratinocytes produce predominantly IL-1 alpha and that this is secreted from the cells *in vitro* (Kupper *et al*, 1986). Schmitt *et al* (1986) have also suggested that the epidermis represents a large pool of IL-1 that may arise in response to continual exposure of keratinocytes to stimuli such

as UV light. This pool could contribute to the high IL-1 alpha levels seen in all individuals. Any further IL-1 alpha produced from activated sites, such as the synovium in arthritis patients, would not result in a significant rise above basal levels. However, consideration of one individual, by longitudinal assessment, would allow detection of small fluctuations in plasma levels corresponding to changes in disease activity.

Recent studies in which plasma IL-1 has been measured, show increased IL-1 beta levels in a number of disease states, including infectious purpura (Girardin *et al*, 1988), experimental endotoxin fever (Cannon *et al*, 1990) and renal transplant recipients (Maury and Teppo, 1988). The IL-1 alpha however appears to remain at levels comparable to those in normal individuals (Cannon *et al*, 1990).

We have shown the benefit of using the chloroform extraction protocol when measuring plasma IL-1 beta but not alpha and, by immunoassay, have demonstrated detectable levels of both cytokines in normal individuals and RA patients. Levels of IL-1 beta could be correlated with disease activity in the RA population and in longitudinal studies. Interleukin 1 alpha was also related to disease activity when serial measurements from individuals were examined.

4. RESULTS: Characterisation of plasma IL-1

4.1. INTRODUCTION

To confirm that the plasma IL-1 immunoreactivity measured corresponded to the presence of bioactive cytokine, plasma was separated by gel filtration before assay. This also enabled a preliminary identification of IL-1 inhibitory activity in the EL4/CTLL costimulator assay.

Early analysis of IL-1 activity in *ex vivo* biological fluids involved an initial fractionation step followed by bioassay. In other studies, this has resulted in the finding of IL-1 bioactivity at high molecular weights. In amniotic fluid Tamatani *et al* (1988) indicate that IL-1 activity was present in fractions eluting between 60-90 kDa and was neutralisable by antisera to the IL-1 beta form only. Concentration of this activity peak followed by rechromatography resulted in IL-1 activity eluting at 15-20 kDa, suggesting that in amniotic fluid, IL-1 beta is associated with a higher molecular weight protein.

During the isolation of IL-1 activity from stimulated cells it has occasionally been noted that the use of serum in culture media results in the appearance of high molecular weight complexes. Furukawa *et al* (1987) have shown IL-1 activity at above 70 kDa in supernatants from a monocytic line. Togawa *et al* (1979) could identify secreted thymocyte stimulatory activity at 50-70 kDa derived from mononuclear cells, and demonstrated that if low molecular weight LAF was added to human serum the activity rechromatographed as the high molecular weight form. Treatment of large intracellular IL-1 species with detergent has resulted in generation of forms with sizes characteristic of the mature molecule (Lepe-Zuniga *et al*, 1985; Kimball *et al*, 1985).

The initial report describing the IL-1 extraction procedure also analysed the molecular sizing of plasma IL-1 beta immunoreactivity and of bioactive material (Cannon *et al*, 1988). This showed that fractionated extracted plasma contained IL-1 beta immunoreactivity at 17 kDa with minor higher peaks at above 150 kDa. Bioactivity analysis also identified a 17 kDa IL-1 molecule and higher molecular weight material. The first bioactive peak co-eluted with the largest immunoreactive moiety. Another study on

fractionated plasma indicated that both IL-1 alpha and IL-1 beta immunoreactivity are present as high molecular weight (>66 kDa) forms (Capper *et al*, 1990).

Many groups have identified inhibitors of IL-1 bioactivity in serum following fractionation studies (Luger *et al*, 1986; Tamatani *et al*, 1988). These usually appear at molecular weights between 50-70 kDa, the specificity of these IL-1 inhibitors is often not well characterised.

We aimed to identify IL-1 bioactivity in extracted and unextracted human plasma using gel filtration. This could then be compared with a similar immunoreactivity profile. This technique would also facilitate the preliminary identification of specific inhibitors of IL-1 alpha and beta in the EL4/CTLL bioassay.

4.2. PLASMA FRACTIONATION

A large scale normal plasma sample (~30ml) was taken and prepared as described previously. Half was then extracted (Sections 2.3.a. and 2.3.d.) and twelve millilitres of extracted or unextracted plasma were chromatographed on a Sephacryl S-200 gel filtration column and 2ml fractions were collected (Section 2.6.).

4.2.a. Protein elution profile

The protein content of individual fractions was determined by measuring the OD at 280nm. Both unextracted and extracted plasma samples gave similar broad protein peaks, eluting immediately after the void volume (Figure 4.2.1.). Extraction considerably reduced the size of the peak, indicating removal of a considerable amount of protein across a broad molecular weight range.

4.2.b. Plasma IL-1 alpha profile

Unextracted plasma showed low IL-1 alpha immunoreactivity (Section

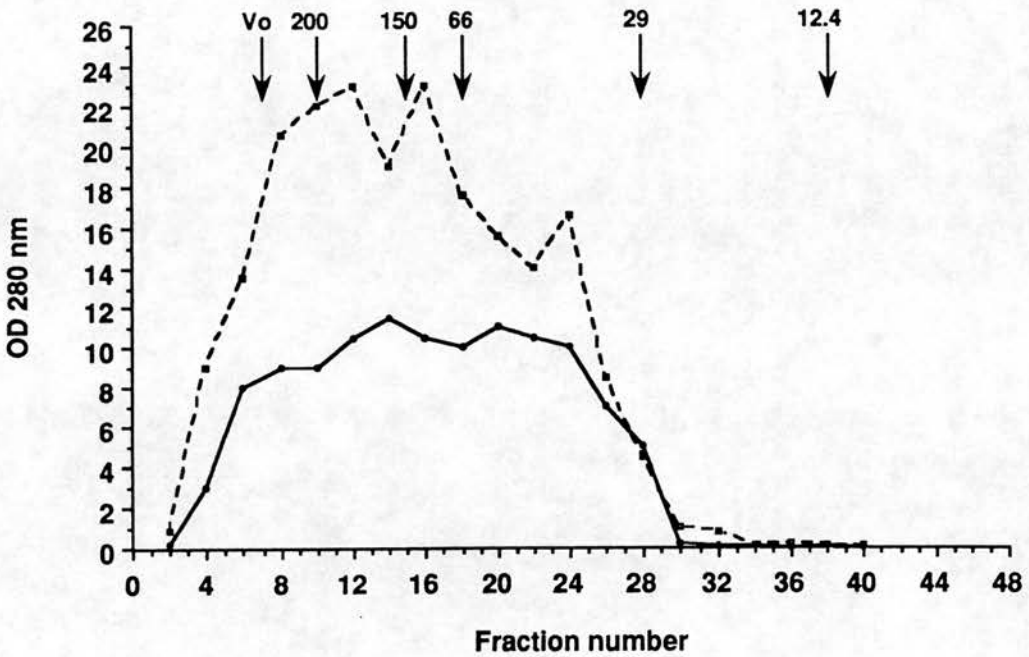

Figure 4.2.1. Plasma protein profile before and after chloroform extraction
The protein profile (OD 280nm) of fractionated normal human plasma before (- - -) and after (——) chloroform extraction is shown. The position of molecular weight markers, expressed in kDa, are indicated.

2.4.b.) following fractionation, small peaks were seen at above 66 kDa and above 150 kDa (Figure 4.2.2.). In extracted plasma a comparable lower molecular weight peak was seen, whereas fractions from the >150 kDa peak were of much higher IL-1 alpha concentration. No immunoreactivity was seen at around 17 kDa. This individual showed a small increase in detectable IL-1 alpha levels in whole plasma from 420 to 460pg/ml following extraction.

4.2.c. Plasma IL-1 beta profile

Unextracted plasma samples showed little IL-1 beta immunoreactivity in the Cistron ELISA (Section 2.4.a.i.). Small peaks were observed migrating above the 12 kDa and 29 kDa markers and within the void volume (Figure 4.2.3.). Extraction, however, resulted in detection of a large peak of IL-1 beta activity that eluted immediately after the void volume, and of a considerable amount of low molecular weight material (<12.4 kDa). A small peak of activity at ~20 kDa (Fraction 33) was apparent. This individual showed <20pg/ml immunoreactive IL-1 beta in whole unextracted plasma, but following extraction the level rose to 61pg/ml.

4.3. PLASMA IL-1 BIOACTIVITY

4.3.a. Antisera inhibition of IL-1 bioactivity

Anti IL-1 alpha and IL-1 beta antisera were tested to determine the titre required to inhibit IL-1 bioactivities in the EL4/CTLL assay (Sections 2.1.b. and 2.1.c.). A 1:1000 dilution of each antiserum or pre-immune serum was tested for its ability to inhibit the response to 100pg/ml of each IL-1. Figure 4.3.1. shows that the antibodies completely neutralised the respective IL-1 species, no cross reactivity was seen and pre-immune serum did not affect the biological response.

4.3.b. Plasma IL-1 bioactivity profile

Column fractions were tested in the IL-1 bioassay as described in Section 2.1.b., the results shown represent samples tested at a concentration of 10%. Unextracted plasma showed minimal IL-1 bioactivity, the largest peak

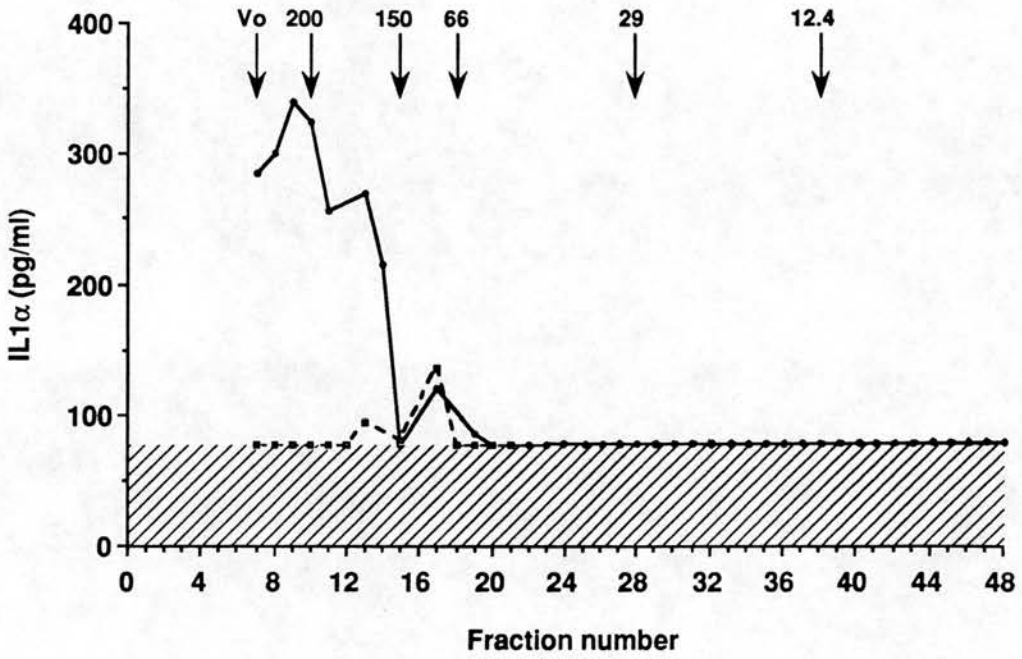

Figure 4.2.2. Plasma IL-1 alpha profile

The IL-1 alpha immunoreactivity profile of fractionated normal human plasma, before (- - -) and after (——) chloroform extraction is shown. The position of molecular weight markers expressed in kDa are indicated. The shaded area represents the detection limit of the assay.

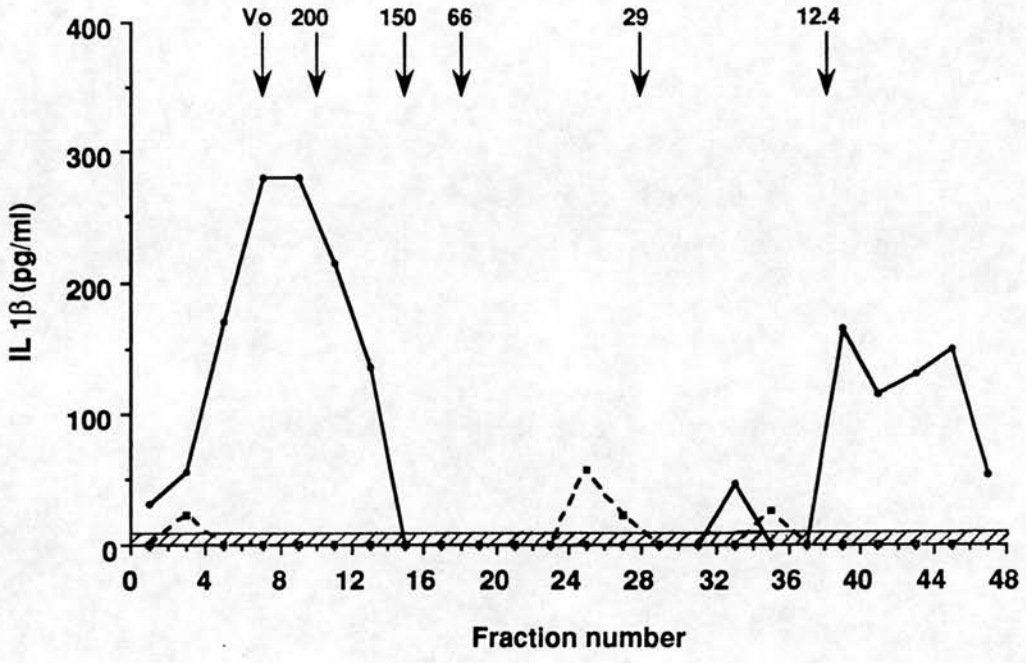

Figure 4.2.3. Plasma IL-1 beta profile

The IL-1 beta immunoreactivity profile of fractionated normal human plasma, before (- - -) and after (——) chloroform extraction is shown. The position of molecular weight markers expressed in kDa are indicated. The shaded area represents the detection limit of the assay.

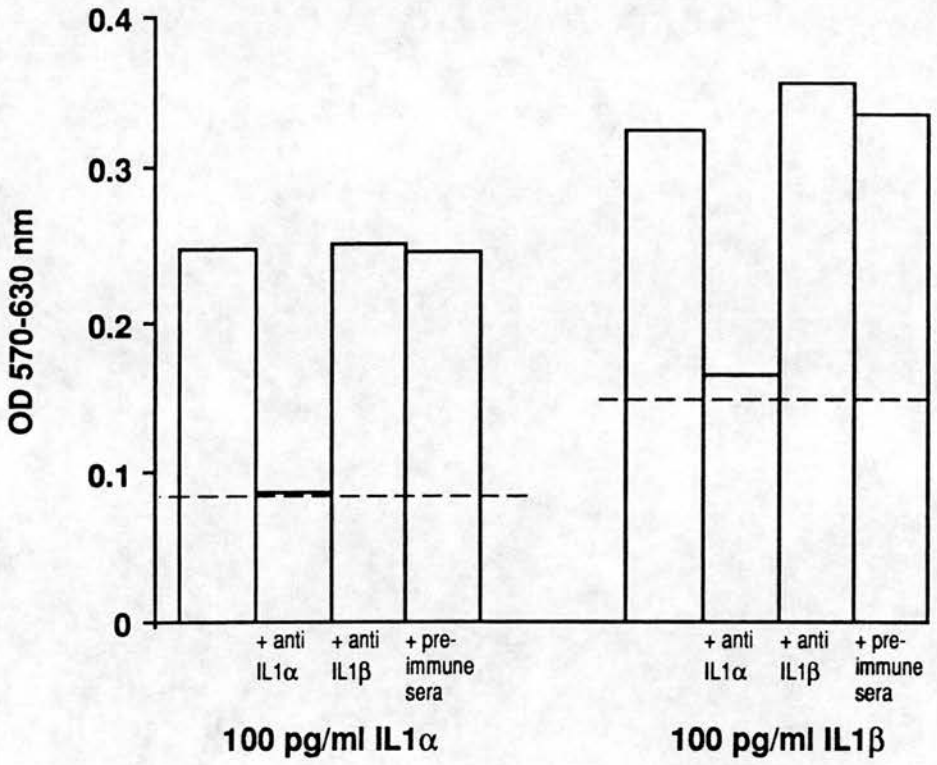

Figure 4.3.1. Anti-sera neutralisation of recombinant IL-1 activity

The neutralisation of 100 pg/ml of IL-1 alpha or beta by anti IL-1 antisera or pre-immune serum at 1:1000 titre is shown. The dashed line represents the background OD.

giving less than 1pg/ml response as compared to standard (Figure 4.3.2.). Extraction identified a bioactive peak at >150 kDa, positive fractions read >100pg/ml compared to recombinant IL-1. All the IL-1 bioactivity shown was completely neutralised by 1:1000 titre of a combination of both anti IL-1 antisera.

4.3.c. Plasma IL-1 inhibitor profile

Unextracted plasma column fractions were incubated at room temperature for 1 hour with 10pg/ml of either IL-1 alpha or beta prior to assay by EL4 cells (Section 2.1.d.). The inhibition profile is shown in Figure 4.3.3.. The results represent the activity of 1% final sample concentration, none of the samples showed cytotoxicity at this concentration. The IL-1 alpha inhibitory activity was never greater than 10%. However inhibition of recombinant IL-1 beta bioactivity showed a peak within the void volume that reduced IL-1 beta bioactivity by 75%.

4.4. DISCUSSION

Fractionation of normal plasma identified little immunoreactive IL-1 alpha or beta unless the sample had previously been chloroform extracted. The procedure revealed high molecular weight IL-1 alpha and beta, the major peak in each case being at >200 kDa. A considerable amount of low molecular weight IL-1 beta was also detected, this may however be due to proteolysis of larger IL-1 species. Bioactivity analysis again identified IL-1 only following extraction. Large molecular weight species were seen with no low molecular weight bioactivity. The IL-1 activity was neutralisable by a combination of antisera to both IL-1 alpha and beta. Chloroform extraction was shown to remove a considerable amount of protein at a range of molecular weights throughout the protein peak.

Specific IL-1 beta inhibitory activity was identified in normal, unextracted plasma at high molecular weights, eluting near the void volume. No corresponding IL-1 alpha inhibitory activity was seen.

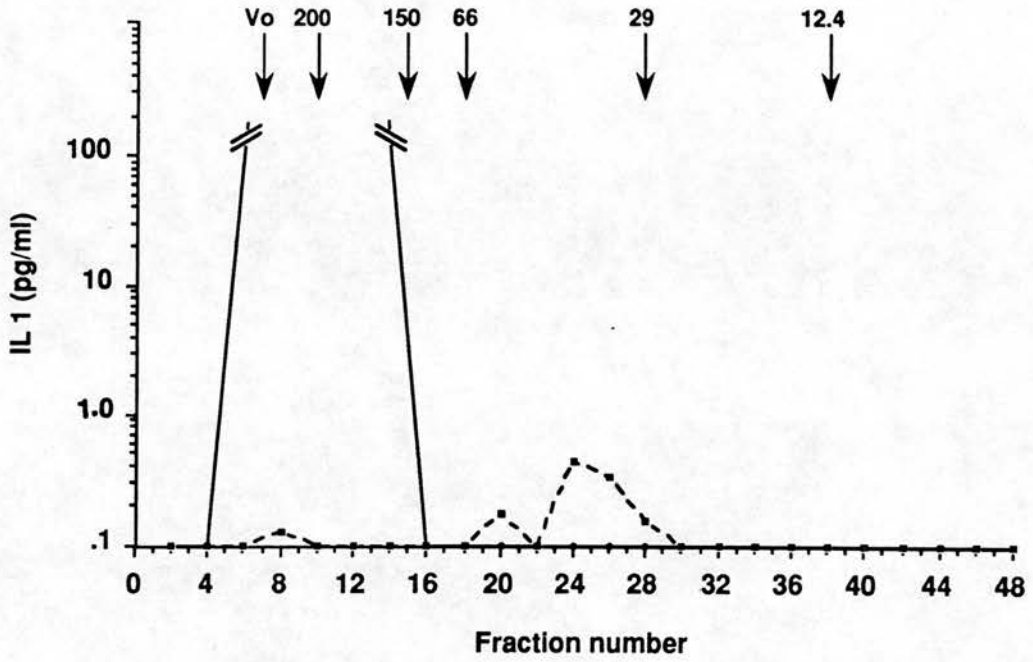

Figure 4.3.2. Plasma IL-1 bioactivity profile

The profile of fractionated plasma IL-1 bioactivity before (- - -) and after (——) chloroform extraction is shown. Samples are all tested at 10% concentration and the activity shown is neutralisable by anti IL-1 antisera. Molecular weight markers in kDa are indicated.

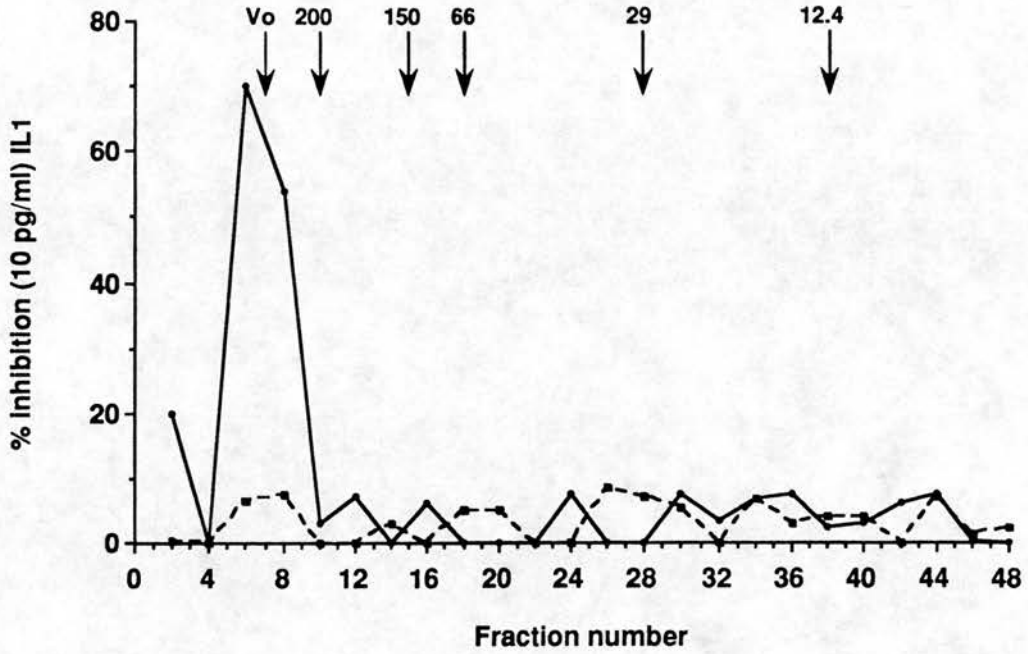

Figure 4.3.3. Plasma IL-1 inhibitor profile

The profile of IL-1 inhibitory activity in unextracted fractionated plasma is shown. The lines represent inhibition of 10 pg/ml of either human recombinant IL-1 alpha (---) or IL-1 beta (——). Samples are tested at 1% concentration. The positions of molecular weight markers in kDa are indicated.

From immunoreactivity data it appears that both IL-1 molecules are present in the circulation as high molecular weight complexes, and that the associated proteins mask epitopes recognised by antibodies in the assays used. Unexpectedly, extraction of plasma revealed high IL-1 alpha concentrations in this individual after fractionation. However examination of plasma showed that in this case chloroform extraction did increase the amount of the detectable IL-1 alpha. Capper *et al* (1990) identify high molecular weight plasma IL-1 alpha without extraction using the same immunoassay; the levels they detected were, however, considerably lower with a peak of 150 pg/ml at >66 kDa. This could correspond to the peak observed in our unextracted plasma run, eluting close to the 66 kDa marker. In monocyte culture supernatants high molecular weight IL-1 alpha (>150 kDa) has been demonstrated when serum is included in culture media (Ferrua *et al*, 1988).

The plasma IL-1 beta profile identified minimal immunoreactivity in unextracted plasma, but a high molecular weight peak was apparent following extraction. Plasma IL-1 beta has also been demonstrated by Capper *et al* (1990) using a different immunoassay system that is not dependent on prior extraction. The study did not, however, find low molecular weight IL-1 beta species, indicating that this material may be a result of proteolytic breakdown of higher molecular weight material during extraction or fractionation. A high molecular weight species (>150 kDa) of IL-1 beta has also been found in the supernatants of blood monocytes cultured in the presence of serum (Ferrua *et al*, 1988).

In contrast to previous studies our bioactivity data indicate that fractionation alone is not sufficient to identify plasma IL-1. This may be due to differences in the assays used. The majority of other reports use the thymocyte proliferation assay known to respond to murine TNF, IL-6, IL-2 and murine IL-4. However the EL-4/CTLL assay does not recognise either human TNF or human IL-6, and when tested on CTLL cells alone positive fractions did not cause proliferation indicating the absence of human IL-2. The use of specific antibodies to both IL-1 forms completely inhibited the IL-1 response. In extracted plasma, high molecular weight IL-1 activity was again detected in

fractions shown to contain immunoreactivity. Interleukin 1 bioactivity was not seen at 17 kDa.

Initial description of the chloroform extraction identified IL-1 beta immunoreactivity at high molecular weights and at 17 kDa (Cannon *et al*, 1988). Pooling and rechromatography of the larger material resulted in the appearance of 17 kDa IL-1. Capper *et al* (1990) showed that IL-1 beta/protein complexes could be dissociated at acid pH to give high levels of immunoreactivity at <30 kDa.

The data suggest that rather than being present as an isolated molecule, circulating IL-1 exists in complexes with other proteins. Binding to a high molecular weight factor (>200 kDa) is seen, this interaction does not appear to affect biological activity. However the association of another molecule, that is removable by chloroform extraction, inhibits biological responses to IL-1 and blocks the recognition of certain epitopes by immunoassay.

The inhibitory effect of fractionated normal plasma on IL-1 bioactivity was assessed, we added exogenous IL-1 to fractions to show specific anti IL-1 effect rather than examining the cellular response to fractions alone. Inhibition of IL-1 beta but not IL-1 alpha bioactivity was demonstrated at high molecular weights (>200 kDa). This may represent the association of IL-1 beta with high molecular weight proteins or masking factors described. Alternatively, this could be the effect of a specific IL-1 inhibitor such as those previously described. If inhibition was a result of IL-1 beta binding to the high molecular weight factors seen in this study, the lack of effect with IL-1 alpha could imply that different moieties mask epitopes on IL-1 alpha and beta, or that IL-1 alpha less readily associates with the available molecules. This could also explain why IL-1 alpha is less consistently affected by extraction than IL-1 beta.

There are various candidate high molecular weight proteins that may act as carriers or inhibitors of IL-1 action. Alpha $_2$ macroglobulin (alpha $_2$M) has been shown to bind to IL-1 beta (Borth and Luger, 1989) and other cytokines (James, 1990). The IL-1 beta/alpha $_2$M complex was shown to migrate at >160 kDa by HPLC molecular sieve chromatography and to retain biological activity in

the thymocyte co-stimulator assay. Complexes were also shown between alpha $_2$M and iodinated IL-1 beta in plasma and serum. Complex formation between alpha $_2$M and IL-6 has been shown to protect the cytokine from the action of a number of proteolytic enzymes, while not altering its receptor binding or biological activity (Matsuda *et al*, 1989). The binding of TGF beta to alpha $_2$M has been shown to be in part acid labile (O'Conner-McCourt and Wakefield, 1987). The sensitivity of high molecular weight IL-1 complexes to acid treatment (Capper *et al*, 1990) suggests that this may represent a similar type of binding.

Autoantibodies to IL-1 alpha have been identified in the sera of about 10% of normal individuals. This is proposed to account for the binding of iodinated IL-1 alpha to 100-200 kDa factors in pooled serum (Svenson *et al*, 1989). Autoantibodies were shown to inhibit the bioactivity of IL-1 alpha in the EL-4 assay. Preliminary studies to identify autoantibodies to IL-1 beta in the course of the work reported here showed some activity in the serum of individuals with systemic lupus erythymatosus (data not shown).

The IL-1 binding factors seen in normal human plasma could serve two purposes: to protect the local and systemic environment from the potentially damaging effects of free IL-1, and to facilitate the cytokine's removal from the circulation. Alpha $_2$M is known to effect the removal of bound proteases. Alternatively, they could serve a carrier function, allowing retention of biological activity and perhaps even protecting IL-1 from the action of proteases in both the circulation and at the local site of inflammation. Radiolabelled IL-1 beta has been shown to clear from plasma in a biphasic manner, with an initial rapid half life and a second slower one of about 4 hours (Klapproth *et al*, 1989). It has also been shown that IL-1 beta associates with the plasma fraction in blood (Newton *et al*, 1988). The second slower clearance phase is consistent with IL-1 associating with a plasma protein.

Our studies show that plasma IL-1 exists in the circulation as complexes with large proteins rather than as the free molecule. This is demonstrated

for IL-1 alpha, beta and the bioactive form. Binding to the high molecular weight material does not prevent the detection of bioactivity, however some factor is present that is able to mask both immunoreactive epitopes and bioactivity. A high molecular weight moiety is also demonstrated in plasma that is able to inhibit the bioactivity of IL-1 beta but not IL-1 alpha.

5. RESULTS: The interleukin 1 beta binding protein

5.1. INTRODUCTION

Local production of IL-1 at sites of inflammation is potentially damaging to surrounding tissue. The extensive tissue destruction often seen at sites of inflammatory disease may be attributed, at least in part, to the effects of local IL-1 synthesis. The presence of circulating IL-1 in normal individuals suggests that factors are present to regulate the *in vivo* effects of the cytokine.

Though high molecular weight factors have been shown to bind plasma IL-1, investigation of the best characterised of these, alpha $_2$ macroglobulin, suggests that association is not sufficient to prevent biological activity (Borth and Luger, 1989; Teodorescu *et al*, 1988). Cytokine autoantibodies may have a role in the regulation of IL-1 effects but are not found in all individuals (Bendtzen *et al*, 1990).

Various inhibitors of IL-1 *in vitro* action have been described (Larrick, 1989). Only one of these has been cloned (Eisenberg *et al*, 1990) allowing study of its *in vivo* effects. This molecule acts by binding to the 80 kDa IL-1 receptor and therefore preventing interaction with any IL-1 present. The inhibitor is produced by monocytes (Hannum *et al*, 1990) and has been identified in both serum and urine (Prieur *et al*, 1987), indicating that it has a role in modulating the effects of circulating IL-1. The physiological importance of the receptor antagonist has been shown by Carter *et al* (1990), who describe its inhibition of IL-1 induced corticosteroid production. An inhibition of IL-1 induced hypotension and acute phase response has also been reported by Ohlsson *et al* (1989).

For a number of other cytokines soluble receptors have been identified, some of which are seen to inhibit biological response to ligand. Soluble receptor forms have been commonly described as constitutive components of serum or urine from normal animals or individuals (Novick *et al*, 1989; Fernandez-Botran and Vitetta, 1990). They have high affinity for ligand and can be shown to inhibit *in vitro* biological responses (Symons *et al*, 1988; Kondo *et al*, 1988).

Though a naturally occurring soluble IL-1 receptor has not been

identified previously expression of a truncated cDNA clone for the 80 kDa molecule produces a protein with high affinity for IL-1 alpha (Dower *et al*, 1989), that is able to inhibit *in vitro* and *in vivo* responses (Maliszewski *et al*, 1990; Fanslow *et al*, 1990).

In this study we aimed to identify IL-1 binding proteins in human *ex vivo* fluids. We report the presence and partial purification of an IL-1 beta binding protein from human plasma and synovial fluid.

5.2. IDENTIFICATION OF IL-1 BINDING FACTORS

5.2.a. Identification by gel filtration

Iodinated IL-1 alpha and beta were added to unextracted and extracted human plasma as described (Sections 2.3.d. and 2.5.a.). Samples were then separated by Sephadex S-200 gel filtration chromatography (Section 2.6.). Figure 5.2.1. shows the association of IL-1 alpha with plasma proteins. In unextracted plasma IL-1 alpha was predominantly found as the free molecule eluting at approximately 17 kDa. However following chloroform extraction IL-1 alpha was seen to associate with a high molecular weight factor at >200 kDa.

When iodinated IL-1 beta was added to untreated plasma a proportion eluted as the free molecule at 17 kDa, but a considerable amount (~6% of the total counts) migrated at a higher molecular weight of ~100 kDa (Figure 5.2.2.). Following chloroform extraction, association of IL-1 beta with a high molecular weight factor was seen within the void volume, this was comparable with the IL-1 alpha binding molecule. Extraction did not appear to alter binding in the 100 kDa complex.

To analyse the specificity of IL-1 association with plasma proteins, the sample was incubated with iodinated IL-1 beta, with or without 100x excess cold IL-1 alpha or beta (Figure 5.2.3.). Two IL-1 binding complexes were seen with iodinated cytokine alone, IL-1 alpha competition did not affect ^{125}I IL-1 beta binding but cold IL-1 beta reduced the amount of binding in the 100 kDa complex (from ~5.1% to ~4.5% of total counts). In plasma preparations, cold competition did not completely eliminate binding in the 100 kDa complex.

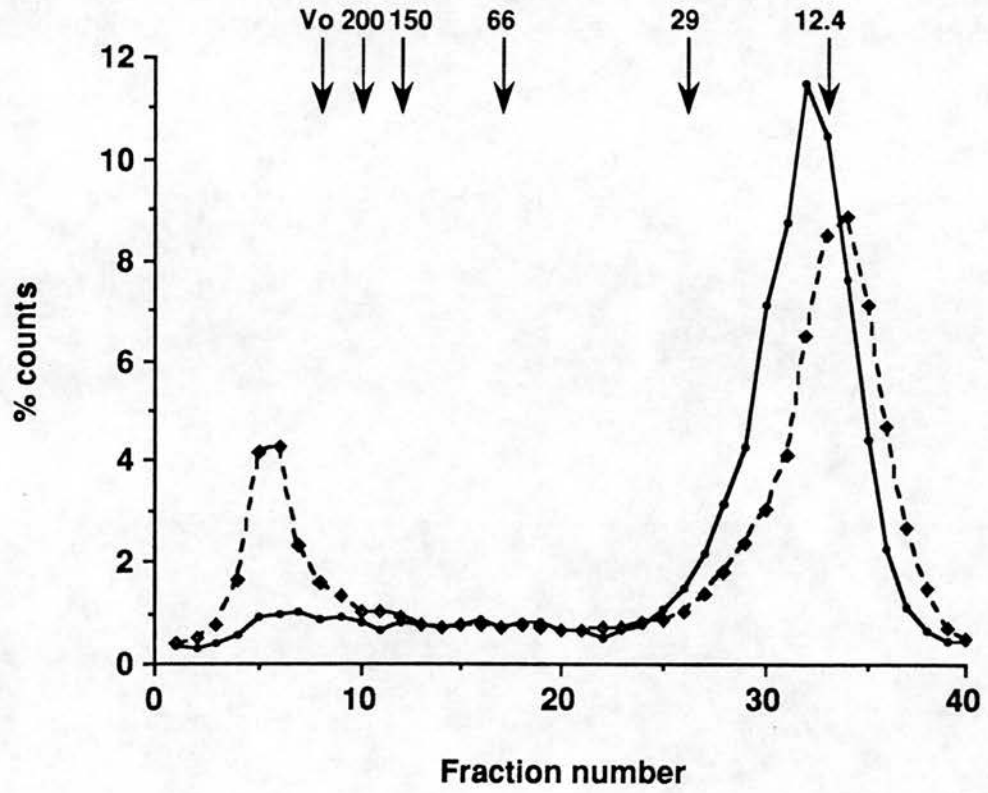

Figure 5.2.1. Gel filtration of IL-1 alpha in human plasma

Extracted (- - -) and unextracted (——) human plasma were incubated with iodinated IL-1 alpha and analysed on a gel filtration column. The percentage of total counts in each 2ml fraction are shown. The position of molecular weight markers in kDa, on the same column, are indicated.

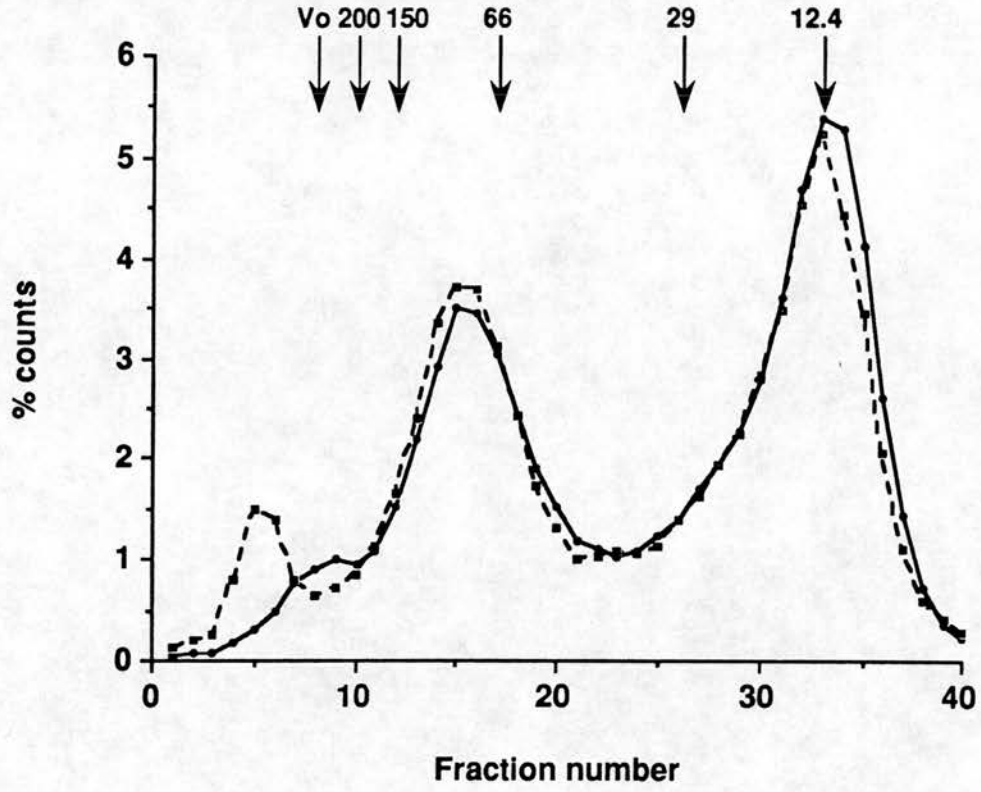

Figure 5.2.2. Gel filtration of IL-1 beta in human plasma

Extracted (- - -) and unextracted (——) plasma were incubated with iodinated IL-1 beta and analysed by gel filtration. The percentage of total counts in each 2ml fraction are shown. The position of molecular weight markers, expressed in kDa, are indicated.

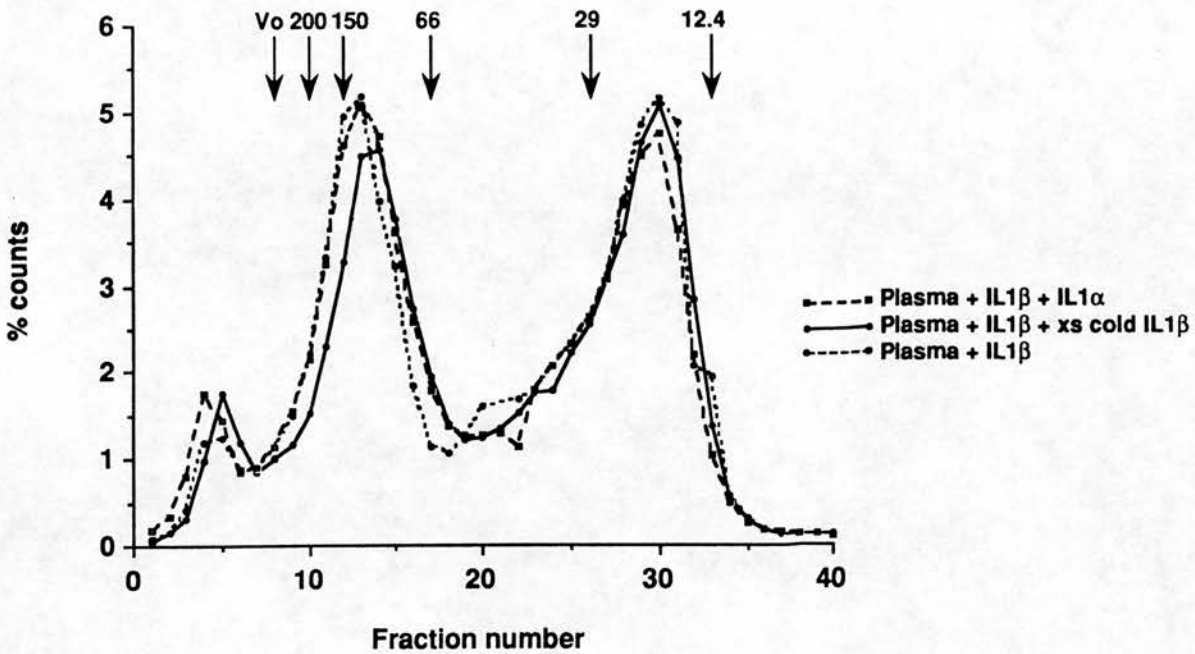

Figure 5.2.3. Specificity of IL-1 beta association with the 100 kDa plasma protein

The binding of iodinated IL-1 beta to plasma proteins is shown (•---•). Specificity of binding was assessed by including 100x excess cold IL-1 alpha (■---■) or beta (——) in the incubation reaction. The percentage of total counts in each 2ml fraction are shown. The position of molecular weight markers in kDa are indicated.

Using synovial fluid samples, however, effective competition of iodinated IL-1 beta by unlabelled IL-1 beta could consistently be demonstrated (Figure 5.2.4., courtesy of J A Symons).

5.2.b. Identification by SDS-PAGE

Plasma samples were incubated overnight with iodinated IL-1, and the associated complexes were cross-linked covalently as described (Section 2.5.b.). Cold competition experiments were done (prior to cross-linking) to examine the specificity of binding. Samples were run on SDS-PAGE gels, as described in Section 2.9.a.. Figure 5.2.5. shows the cross-linking of iodinated IL-1 alpha (A) and beta (B) to plasma samples. Minimal binding was seen with IL-1 alpha, the cytokine being associated with high molecular weight material (>200 kDa). However iodinated IL-1 beta showed specific cross-linking to a complex migrating at about 60 kDa. Binding was competable by excess cold IL-1 beta but not IL-1 alpha or TNF. High molecular weight IL-1 beta binding was also seen at >200 kDa, this appeared to be partially competed by each cold cytokine.

A comparable gel showing the IL-1 beta binding proteins present in synovial fluid is shown in Figure 5.2.6. (courtesy of J A Symons). Cross-linking again shows a band at about 60 kDa indicating a protein of approximately 43 kDa that binds specifically to IL-1 beta.

The binding characteristics of the 43 kDa protein using a second cross-linking reagent are shown in Figure 5.2.7.. The use of EGS identified a similar molecular weight complex with iodinated IL-1 beta, that could be competed by excess cold IL-1 beta but not alpha.

5.2.c. Binding of IL-1 alpha to cell surface receptors

To confirm that the iodinated IL-1 alpha preparation retained receptor binding ability, the cytokine was cross-linked to surface receptors on 3T3 cells as described in Section 2.5.c.. The results are shown in Figure 5.2.8.. Interleukin 1 alpha was able to bind to 3T3 receptors resulting in a complex that migrated at approximately 97 kDa. Binding was competable with excess

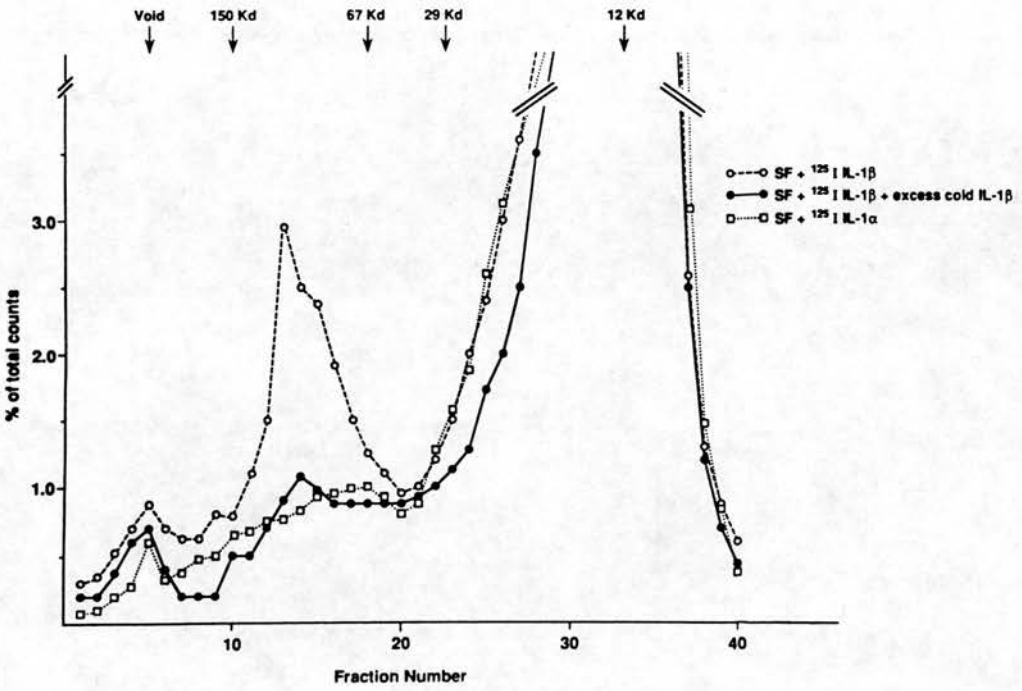

Figure 5.2.4. The association of labelled IL-1 alpha and IL-1 beta with synovial fluid proteins

The binding of iodinated IL-1 alpha (▫...▫) or IL-1 beta (○---○) to synovial fluid proteins is shown. Specificity was assessed by including 100x excess cold IL-1 beta (●——●) in the incubation reaction. The percentage of total counts are shown. The figure was provided courtesy of J A Symons.

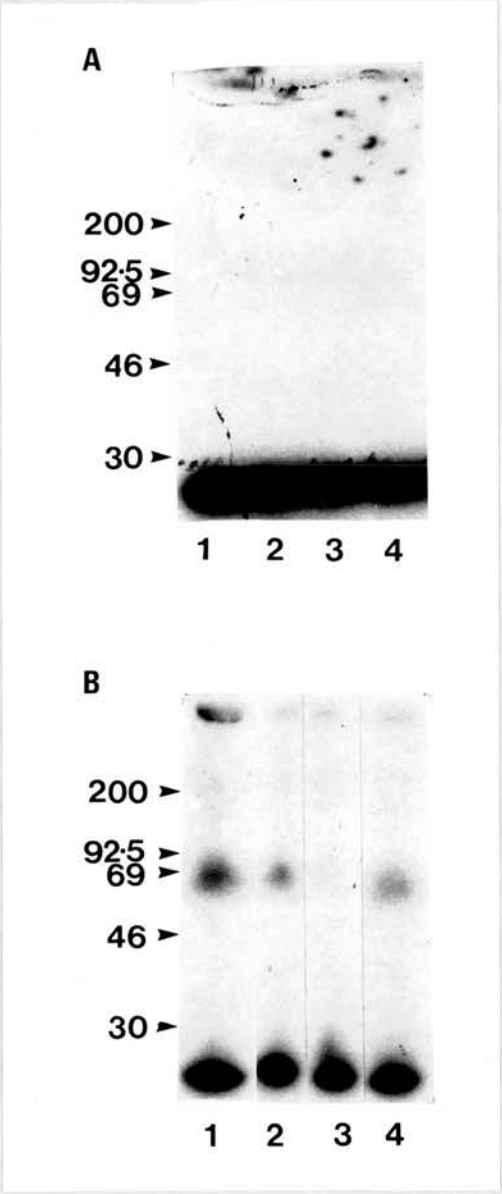

Figure 5.2.5. Analysis of plasma IL-1 binding proteins by SDS-PAGE

Plasma was cross-linked to either iodinated IL-1 alpha (A) or beta (B) and the labelled complexes were analysed by SDS-PAGE (lane 1). The specificity was determined by addition of 100 fold excess of cold IL-1 alpha (lane 2), IL-1 beta (lane 3), or TNF alpha (lane 4). The position of molecular weight markers in kDa are indicated.

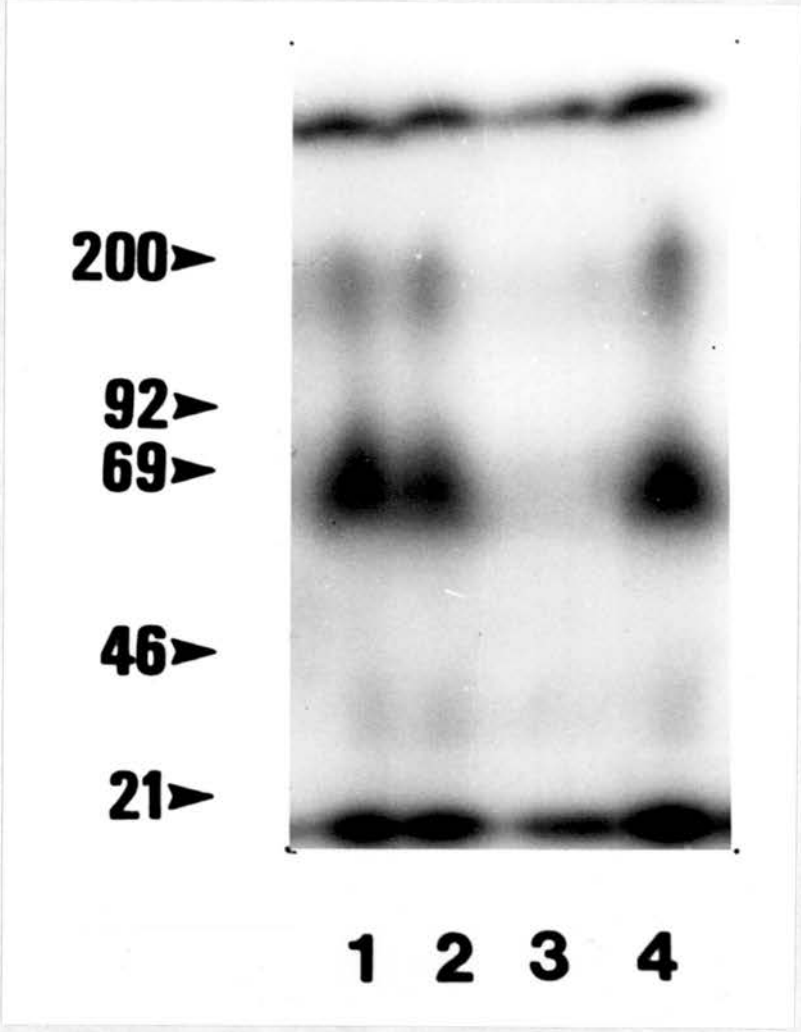

Figure 5.2.6. Analysis of synovial fluid IL-1 binding proteins by SDS-PAGE

Synovial fluid was cross-linked to iodinated IL-1 beta, the products were analysed using SDS-PAGE (lane 1). Specificity was determined by the addition of 100 fold excess of cold IL-1 (lane 2), beta (lane 3) or TNF alpha (lane 4). The figure was provided courtesy of J A Symons. The positions of molecular weight markers in kDa are indicated.

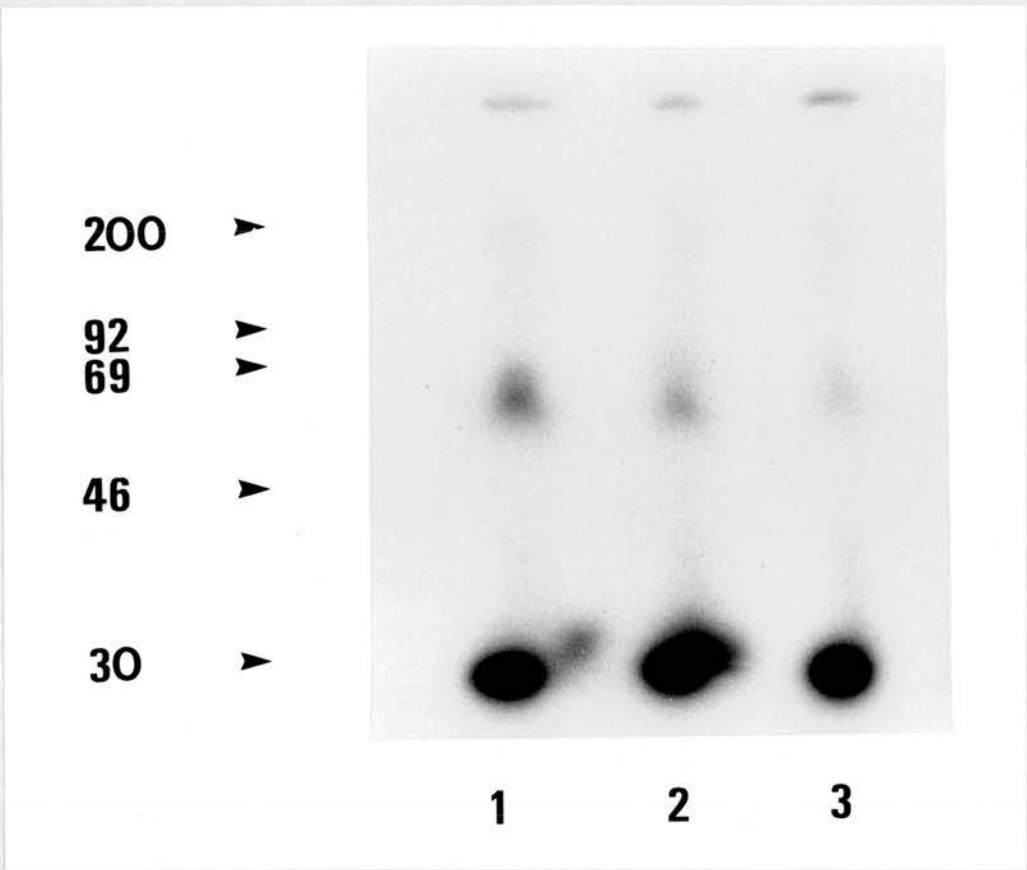

Figure 5.2.7. Analysis of plasma IL-1 beta binding proteins using EGS as the cross-linking agent

The binding and specificity of association of iodinated IL-1 beta with plasma proteins was compared using ethylene glycolbis(succinimidyl succinate) as the cross-linking reagent. Lane 1 shows the binding of IL-1 beta alone and lanes 2 and 3 show competition with excess cold IL-1 alpha and beta respectively. The positions of molecular weight markers in kDa are shown.

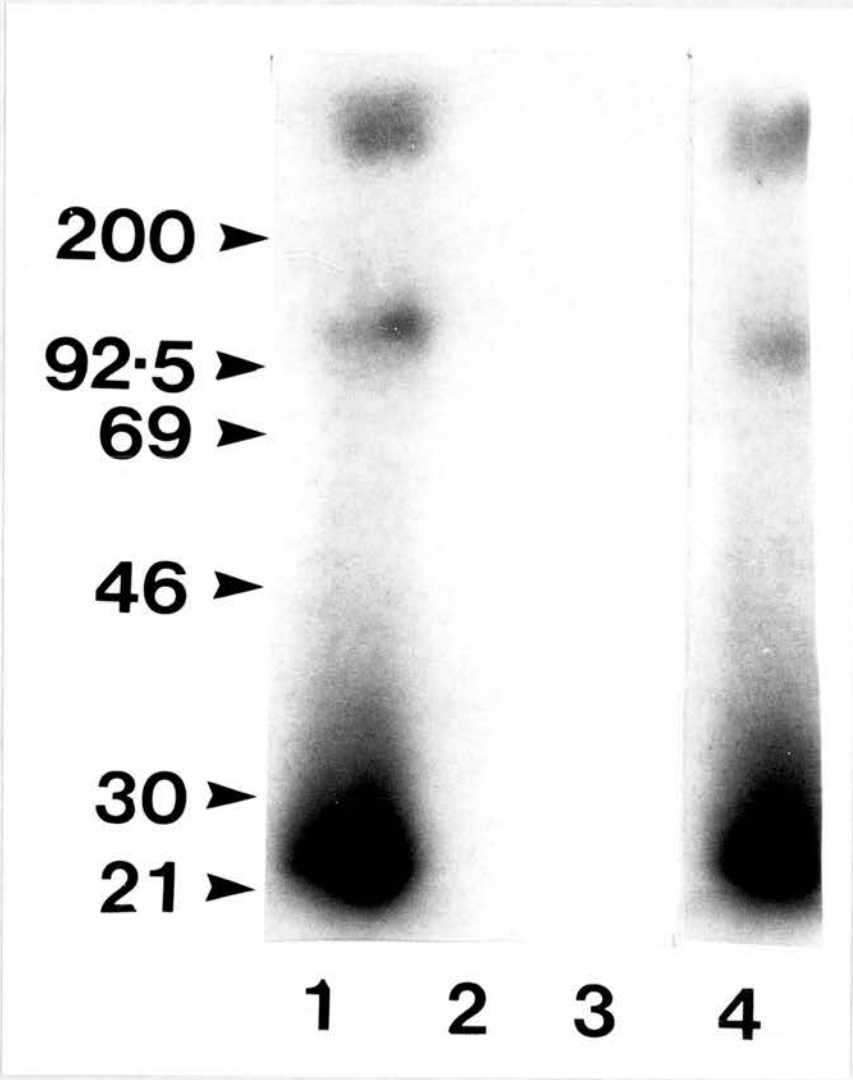

Figure 5.2.8. Cross-linking of IL-1 alpha to the 3T3 cell line

The ability of iodinated IL-1 alpha to bind to the IL-1 receptor on 3T3 cells is shown (lane 1). Specificity is shown by the addition of excess cold IL-1 alpha (lane 2), beta (lane 3) and TNF alpha (lane 4). The position of molecular weight markers in kDa are indicated.

cold IL-1 alpha and IL-1 beta but not with TNF alpha. Thus the iodinated IL-1 alpha bound the cell surface 80 kDa receptor.

5.2.d. Analysis of IL-1 beta binding activities

Fractions containing the 100 kDa IL-1 beta binding protein from Sephadex S-200 column runs were pooled, cross-linked with DSS, concentrated (Section 2.3.c.) and analysed by SDS-PAGE (Figure 5.2.9.). The products of two independent column runs are presented. Both show that the 100 kDa gel filtration complex migrates at approximately 60 kDa in non-reducing polyacrylamide gels.

5.3. PURIFICATION OF THE IL-1 BETA BINDING PROTEIN

5.3.a. Wheat germ agglutinin column

Partial purification of the IL-1 beta binding protein was achieved using a wheat germ agglutinin column. The plasma sample was loaded and eluted from the column as described in Section 2.7.a.. The protein elution profile is shown in Figure 5.3.1.. The majority of the protein did not bind to the column or was eluted in the initial wash steps. Specific elution using N-acetylglucosamine recovered a small protein peak. The binding protein was identified in Fractions 90-92.

Figure 5.3.2. shows the elution of the binding protein from the column by N-acetylglucosamine. Fraction 1 represents the start of the elution step. Fractions were incubated with iodinated IL-1 beta and cross-linked as before.

Figure 5.3.3. shows the binding characteristics of the eluted protein. Fraction 8 from the previous figure was incubated with labelled IL-1 alpha (A) or beta (B) with excess cold cytokine to test specificity. Reducing conditions did not alter the mobility of the binding protein by SDS-PAGE. The results demonstrate that the protein only associates with iodinated IL-1 beta and that IL-1 alpha does not compete for binding.

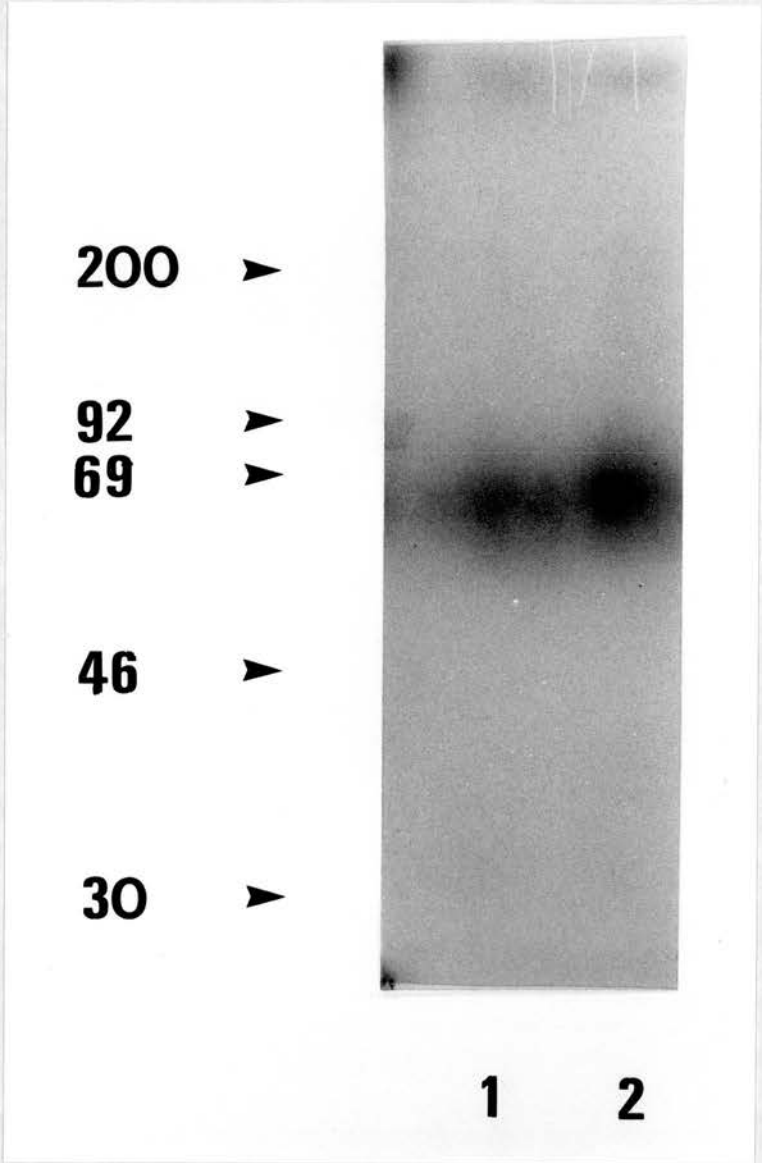

Figure 5.2.9. The migration on SDS-PAGE of the 100 kDa iodinated peak from gel filtration

The 100 kDa peak isolated by gel filtration was cross-linked, concentrated and analysed by SDS-PAGE. Lanes 1 and 2 represent pooled fractions from two separate column runs. Molecular weight markers in kDa are shown.

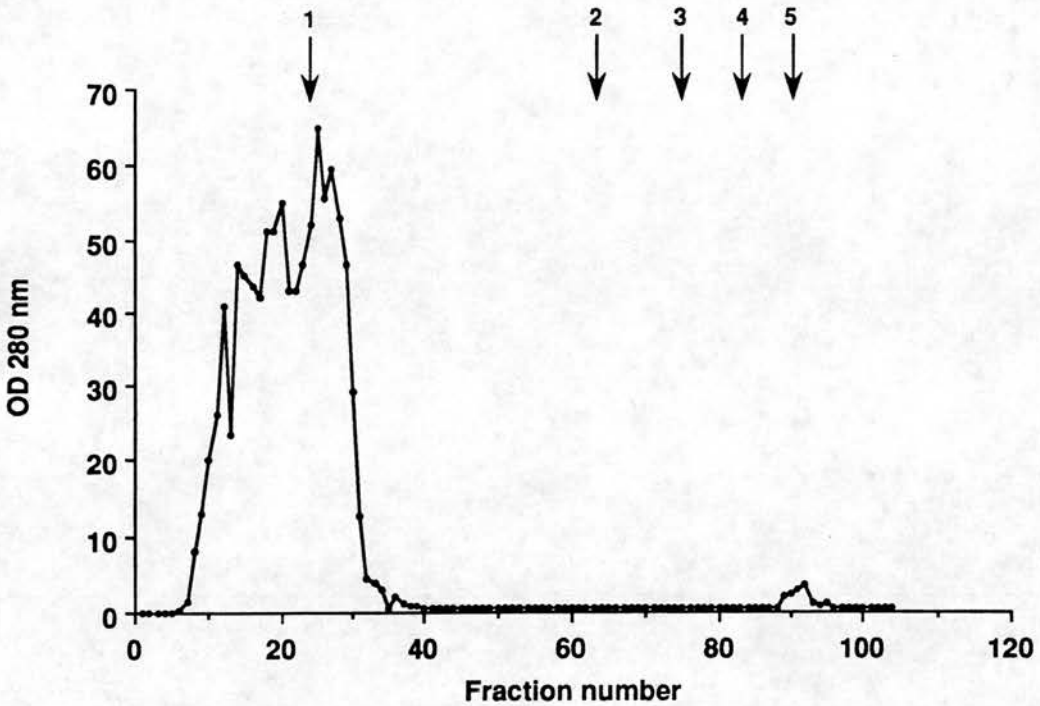

Figure 5.3.1. Protein profile of plasma eluted from a lectin affinity column

The OD at 280nm of plasma fractions collected from a wheat germ agglutinin column were measured. Arrow 1 represents the start of the salt wash, 2 the ethylene glycol wash, 3 PBS and 4 the N-acetyl glucosamine elution step. Arrow 5 marks the peak of binding protein activity.

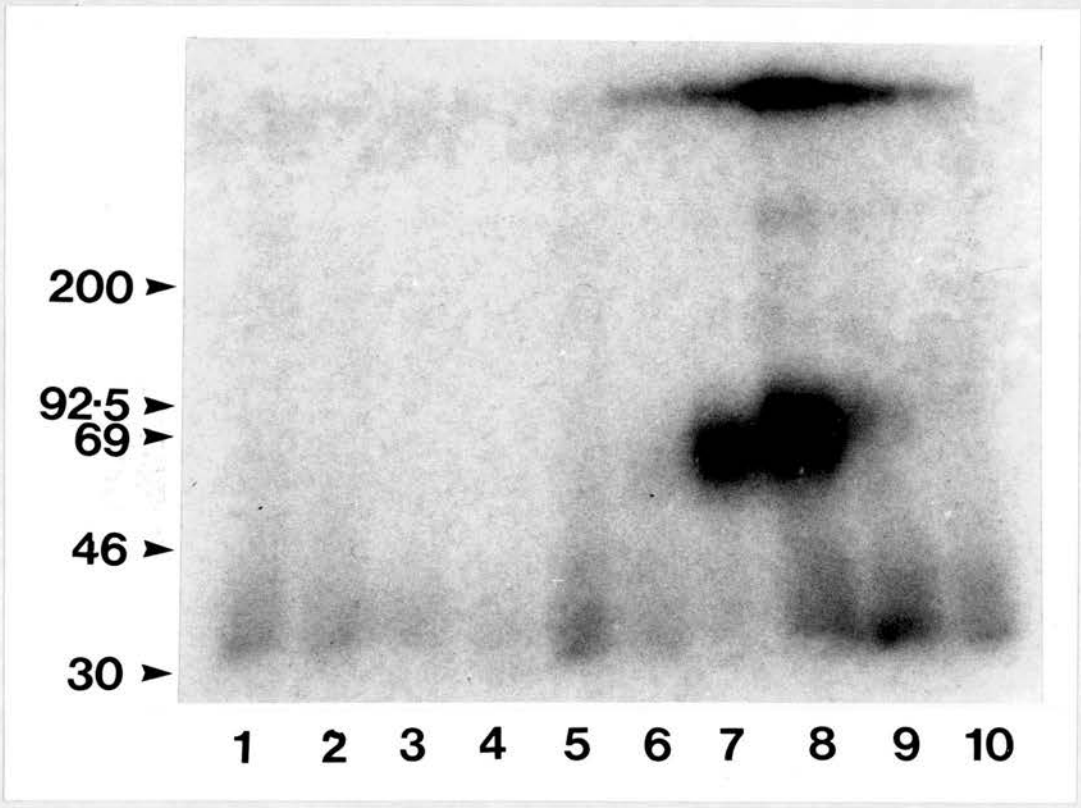

Figure 5.3.2. The IL-1 beta binding activity eluted from the lectin affinity column

Iodinated IL-1 beta was cross-linked to fractions from the N-acetyl glucosamine elution from the wheat germ agglutinin affinity column. The fractions represent numbers 84-93 on the previous figure. Molecular weight markers in kDa are shown.

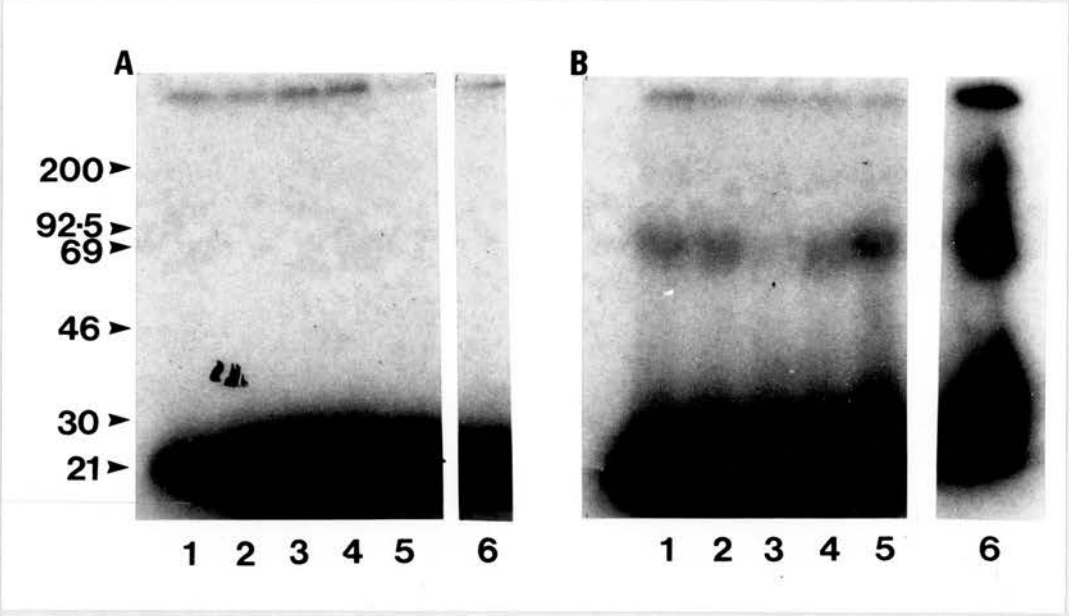

Figure 5.3.3. Specificity of association of IL-1 with the lectin affinity purified binding protein

Iodinated IL-1 alpha (A) and beta (B) was cross-linked to the wheat germ agglutinin purified binding protein (lane 1). Lanes 2, 3, and 4 show the effect of cold competition with IL-1 alpha, beta and TNF alpha respectively. Lane 5 shows the protein run under reducing conditions, and lane 6 a comparable plasma sample. Molecular weight markers in kDa are shown.

5.3.b. Ion exchange purification

5.3.b.i. Binding and elution characteristics of IL-1 beta binding protein

Before purification the conditions required for binding to and elution from DEAE Sephacel were determined. The pH at which the protein bound to the matrix was first established (Section 2.7.b.). Synovial fluid diluted with Tris-HCl at different pH values was incubated with DEAE Sephacel and then tested to determine whether binding protein had been removed from the supernatant. Figure 5.3.4. shows the levels remaining in synovial fluid following incubation with DEAE Sephacel. The results show that at pH 5.0 the protein is still present in the supernatant, whereas at pH 5.5 no 60 kDa complex is evident.

The salt concentration required for elution of protein was then determined. As described in Section 2.7.b. DEAE Sephacel was washed in starting buffer and then equilibrated at a range of salt concentrations. Synovial fluid was added, and the supernatant assayed for binding activity. As shown in Figure 5.3.5. the 60 kDa protein bound to the column at concentrations below 0.1M saline, but above this concentration all activity was recovered in the supernatant.

Therefore 0.01M Tris-HCl at pH 5.5 was selected as the starting buffer and a continuous elution gradient of 0.01-0.5M NaCl was used.

5.3.b.ii. Purification of the IL-1 beta binding protein

The elution profile of the IL-1 beta binding protein, recovered from 3 litres of synovial fluid in 500ml aliquots, is shown in Figure 5.3.6.. The factor eluted as two distinct bands (shaded) at salt concentrations of 0.15 and 0.25M. The cross-linking of associated bands is shown in Figure 5.3.7.. Active fractions were then pooled, concentrated and used for further purification or characterisation.

5.3.c. Affinity purification

An IL-1 beta affinity column was prepared using a modified IL-1 molecule in which a lysine residue was substituted for a cysteine, allowing the

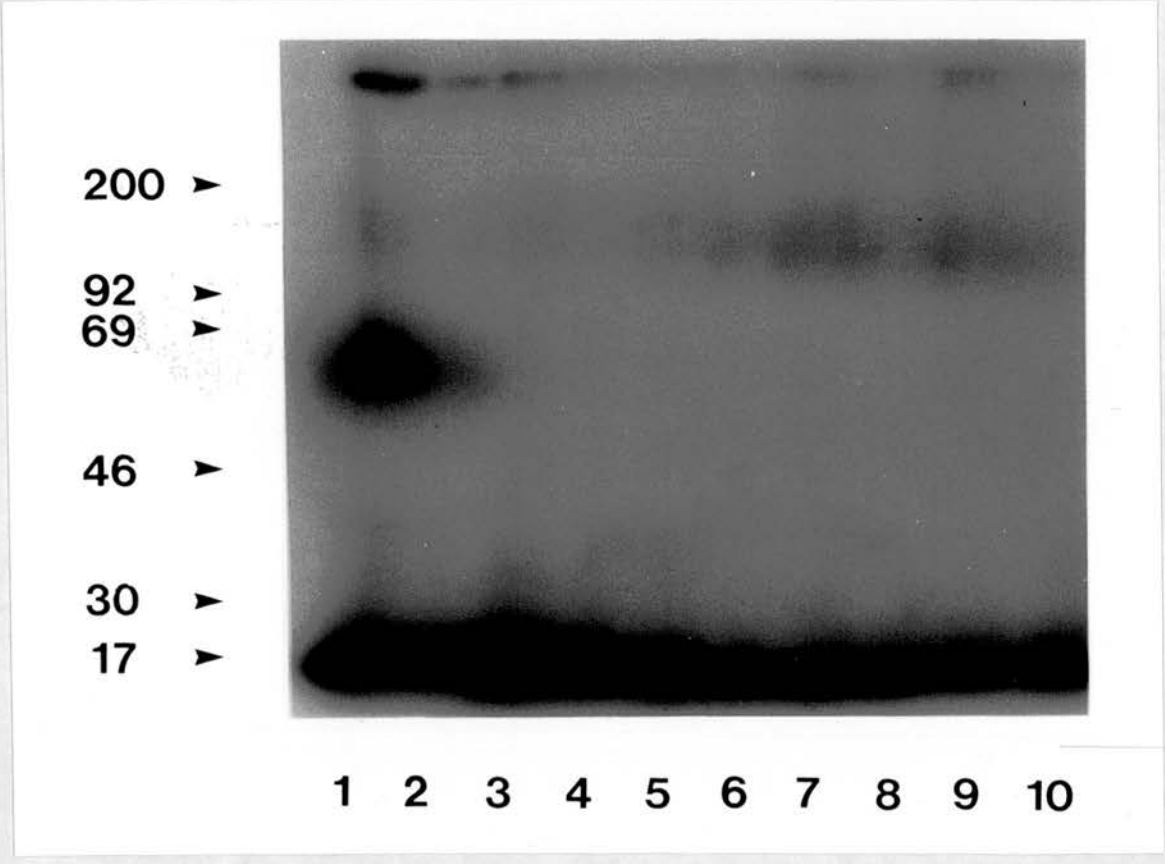

Figure 5.3.4. The effect of pH on the association of binding protein with DEAE Sephacel

The supernatant from synovial fluid incubated with DEAE Sephacel at a range of pH values was tested for loss of binding protein activity. Lane 1 shows whole synovial fluid, and lanes 2-10 supernatants removed from the Sephacel following incubation at a pH range of 5.0-9.0 at intervals of 0.5 pH units. Molecular weight markers in kDa are shown.

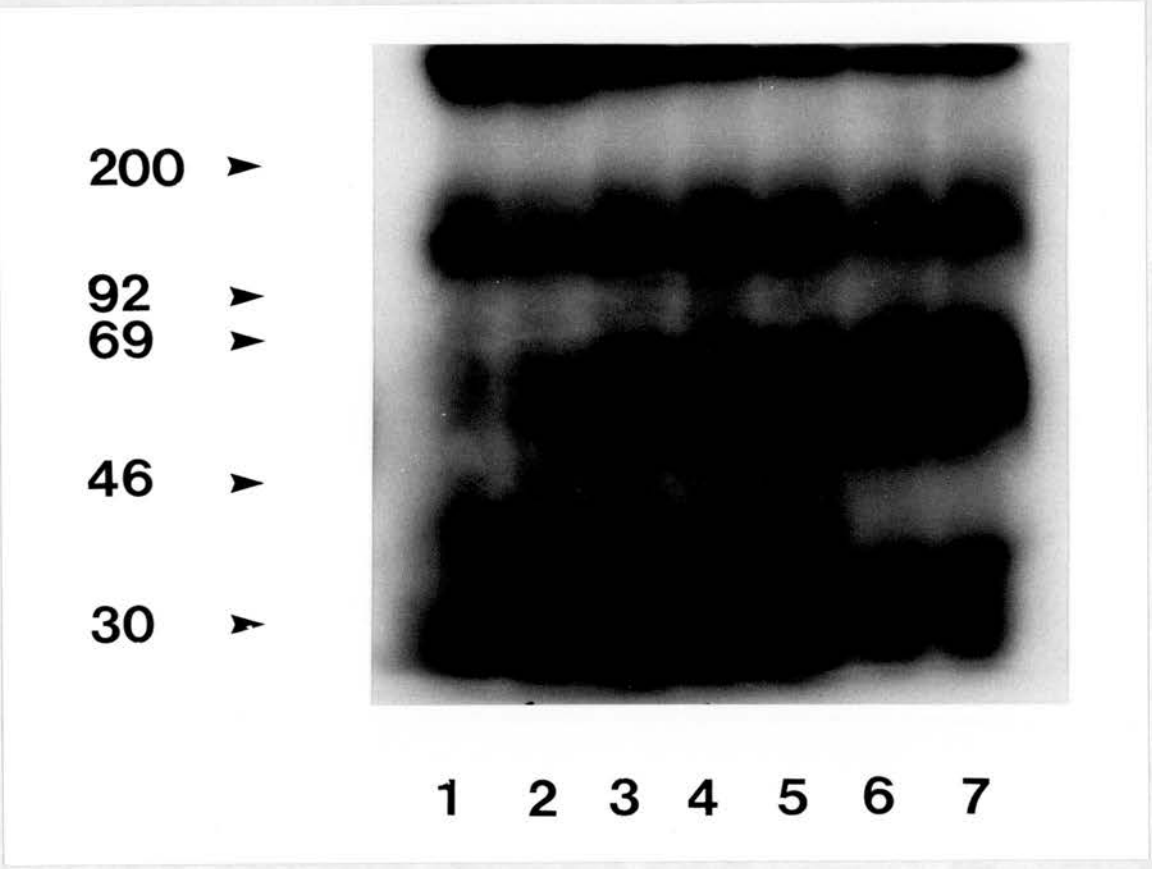

Figure 5.3.5. The effect of salt concentration on association of binding protein with DEAE Sephacel

The supernatant from synovial fluid incubated with DEAE Sephacel at increasing salt concentrations was tested for IL-1 beta binding activity. Lanes 1-7 represent incubation at 0.01, 0.05, 0.1, 0.15, 0.2, 0.25 and 0.3M sodium chloride concentrations. Molecular weight markers in kDa are shown.

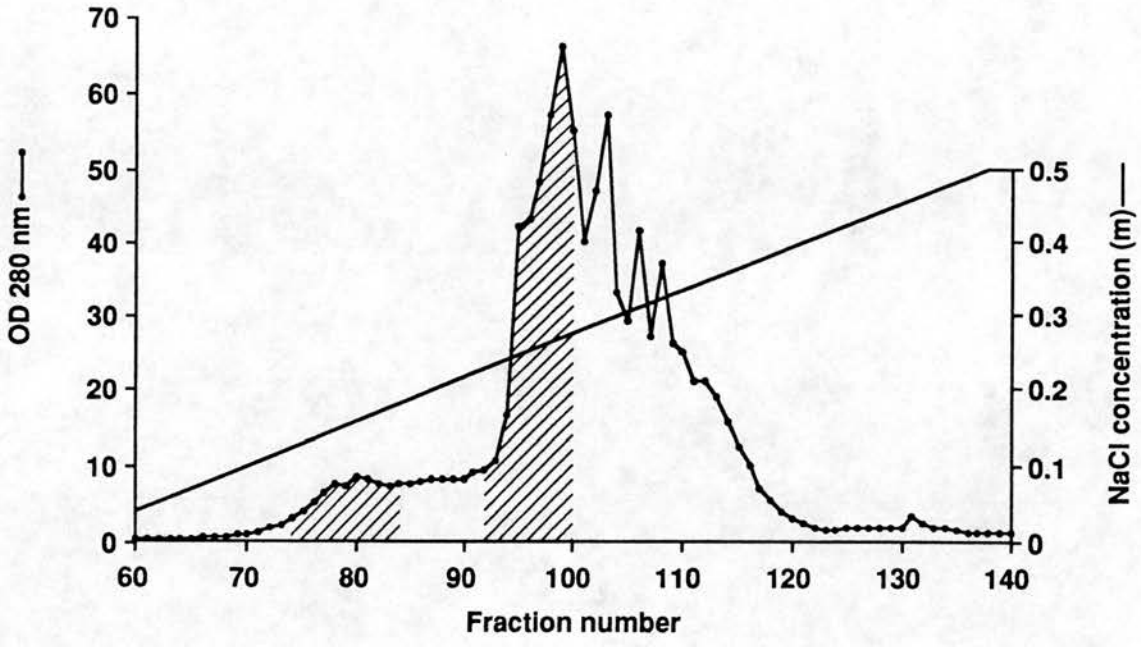

Figure 5.3.6. The protein elution profile from a DEAE Sephacel column

The OD at 280nm of fractions eluting from the ion exchange column are shown, and the salt concentration gradient indicated. Fractions showing IL-1 beta binding activity are represented by the shaded areas.

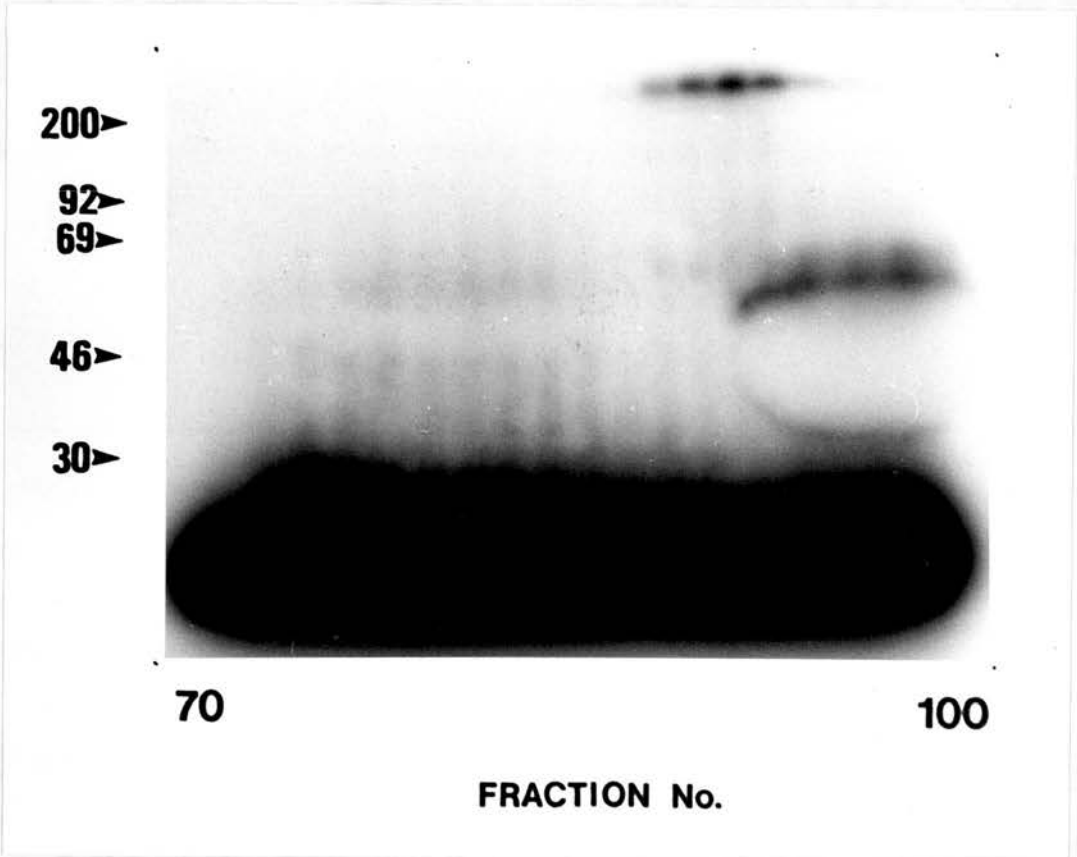

Figure 5.3.7. IL-1 beta binding activity of fractions eluted from the ion exchange column

The cross-linking of IL-1 beta to alternate fractions from the ion exchange column are shown. Positions of molecular weight markers in kDa are indicated.

formation of disulphide bonds with an activated Thiol Sepharose column. Coupling using the protocol described (Section 2.7.c.), resulted in 97% efficiency of binding.

The elution profile of binding protein from the affinity column is shown in Figure 5.3.8.. Binding protein was not eluted by the high stringency salt wash, but strong binding activity was found in the fractions at low pH when coming off the column. Binding specificity in these fractions is also shown, formation of iodinated complex is effectively competed only by cold IL-1 beta.

5.3.d. Reverse phase HPLC of partially purified binding protein

The elution of binding protein with a 10-70% acetonitrile gradient is shown in Figure 5.3.9.. The fractions exhibiting activity by cross-linking on SDS-PAGE gels are shown.

5.4. CHARACTERISATION OF THE IL-1 BETA BINDING PROTEIN

5.4.a. IL-1 beta binding kinetics

The kinetics of association of iodinated IL-1 with the plasma binding protein were examined at 4° C and room temperature (Section 2.5.d.i.). Figures 5.4.1. and 5.4.2. show the respective results. At 4° C little binding was seen in the first three hours, however, overnight incubation at the same temperature resulted in high levels of complex formation. At room temperature considerable binding occurred within the first hour. The sample treated overnight showed much greater binding to the >200 kDa high molecular weight material.

5.4.b. Temperature sensitivity of IL- beta binding protein

Plasma samples were heated to the described temperatures (Section 2.5.d.ii.) before overnight incubation at 4° C with iodinated IL-1 beta. Association of the 60 kDa complex was not greatly affected by heating, only at 70° C was there any reduction of the intensity of the cross-linked band (Figure 5.4.3.). The high molecular weight band showed greater IL-1 beta

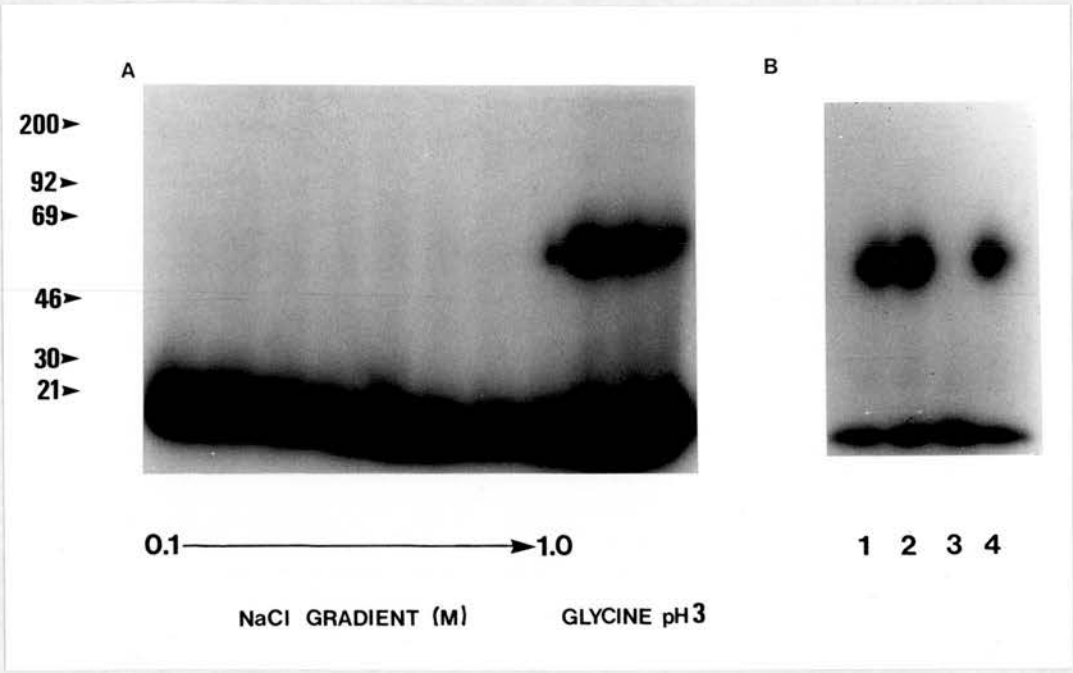

Figure 5.3.8. Elution of binding protein from the IL-1 beta affinity column

Fractions from the salt wash and acid glycine elution, from the immobilised IL-1 beta column, are assessed for binding protein (A). The specificity of binding is also demonstrated (B), lane 1 shows association with iodinated IL-1 beta alone, and lanes 2, 3 and 4 competition with cold IL-1 alpha, beta and TNF respectively. Molecular weight markers in kDa are shown.

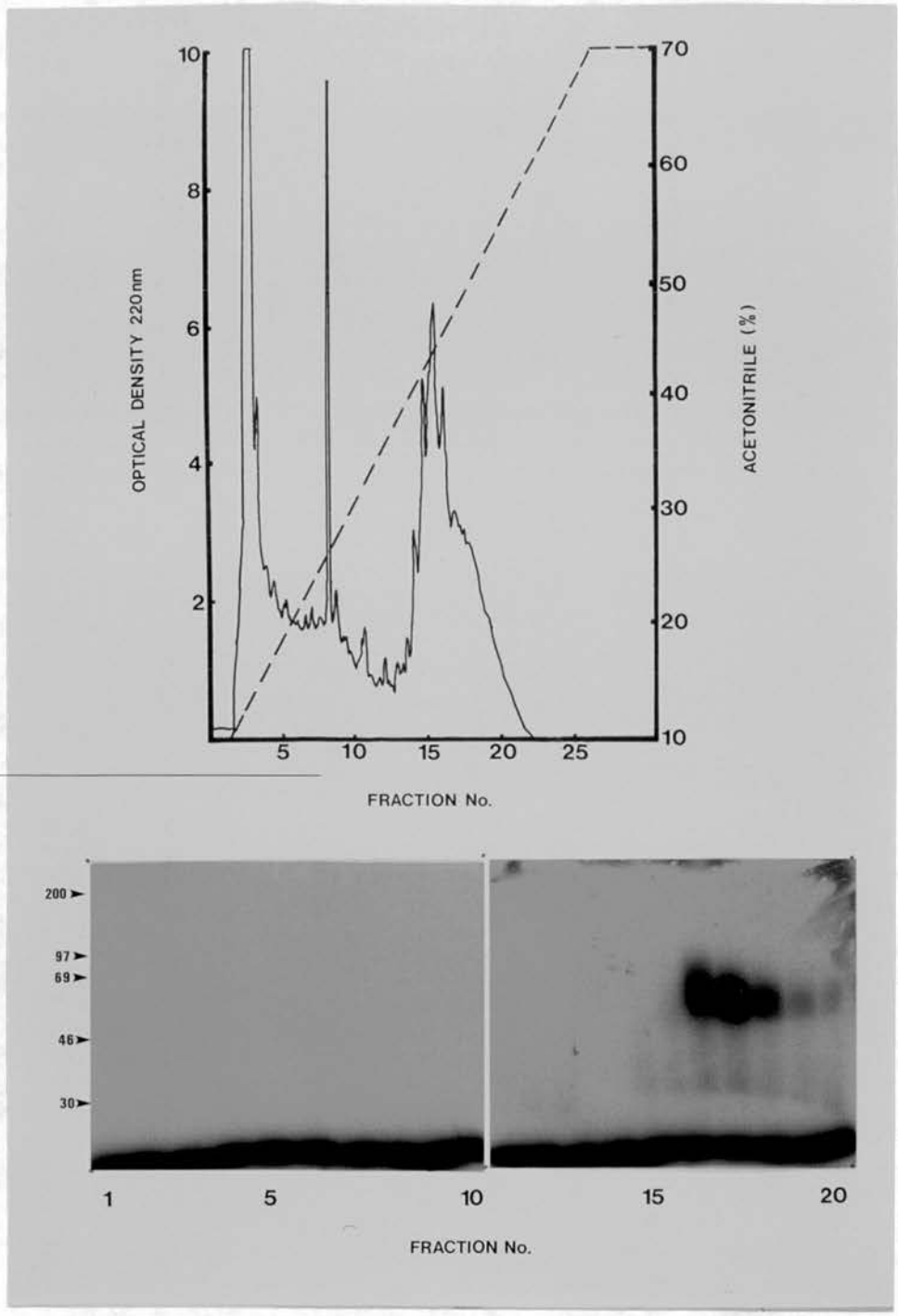

Figure 5.3.9. HPLC analysis of IL-1 beta binding protein activity

The elution profile from the HPLC column, protein OD at 220nm in fractions was determined. The cross-linking of iodinated fractions is shown. The position of molecular weight markers in kDa are indicated.

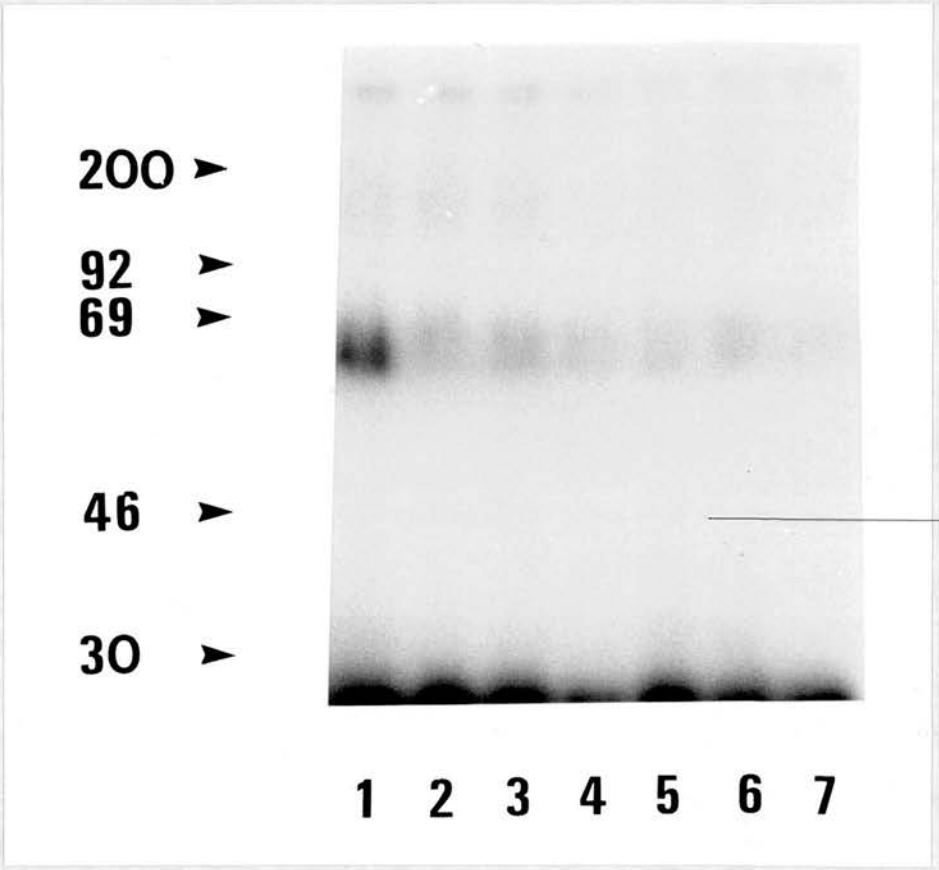

Figure 5.4.1. The kinetics of association of IL-1 beta with the binding protein at 4°C

The association of iodinated IL-1 beta with the binding protein is shown after different incubation times at 4° C. Lanes 1-7 represent incubation overnight, for 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 hours. Molecular weight markers in kDa are shown.

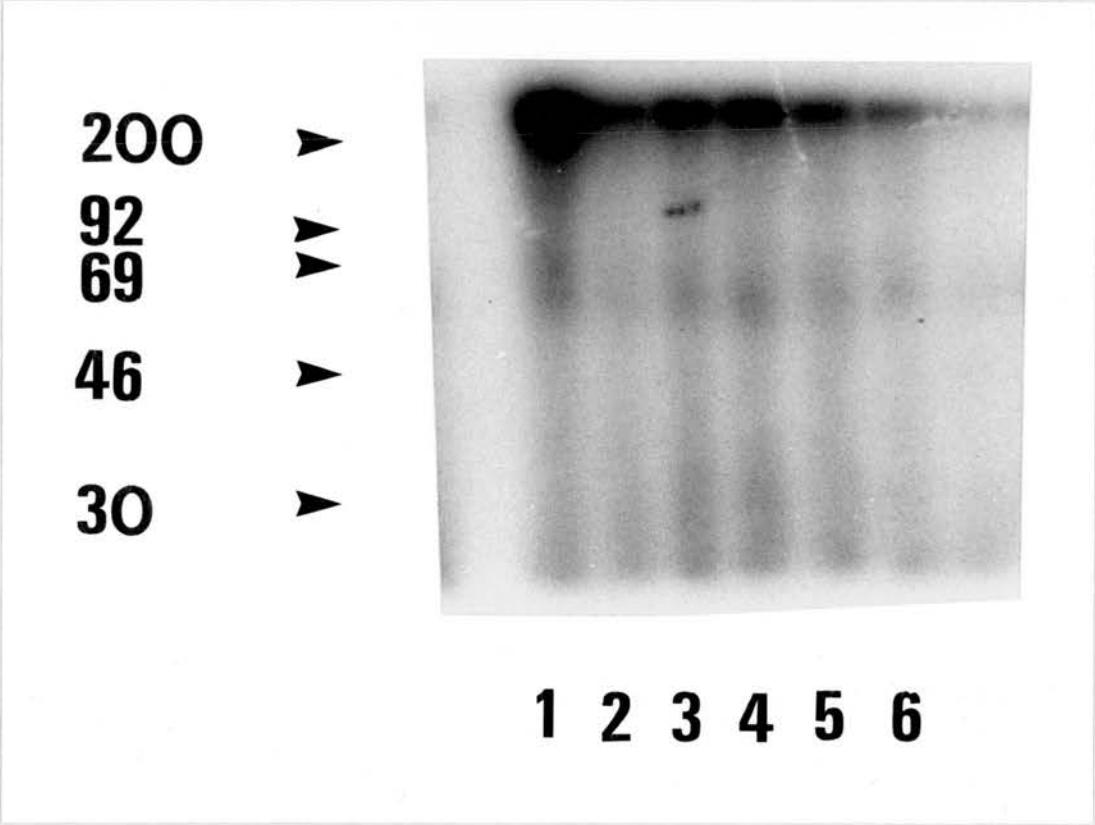

Figure 5.4.2. The kinetics of association of IL-1 beta with the binding protein at room temperature

The association of iodinated IL-1 beta with binding protein is shown after different incubation times at room temperature. Lanes 1-7 represent incubation overnight, for 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 hours. Molecular weight markers in kDa are shown.

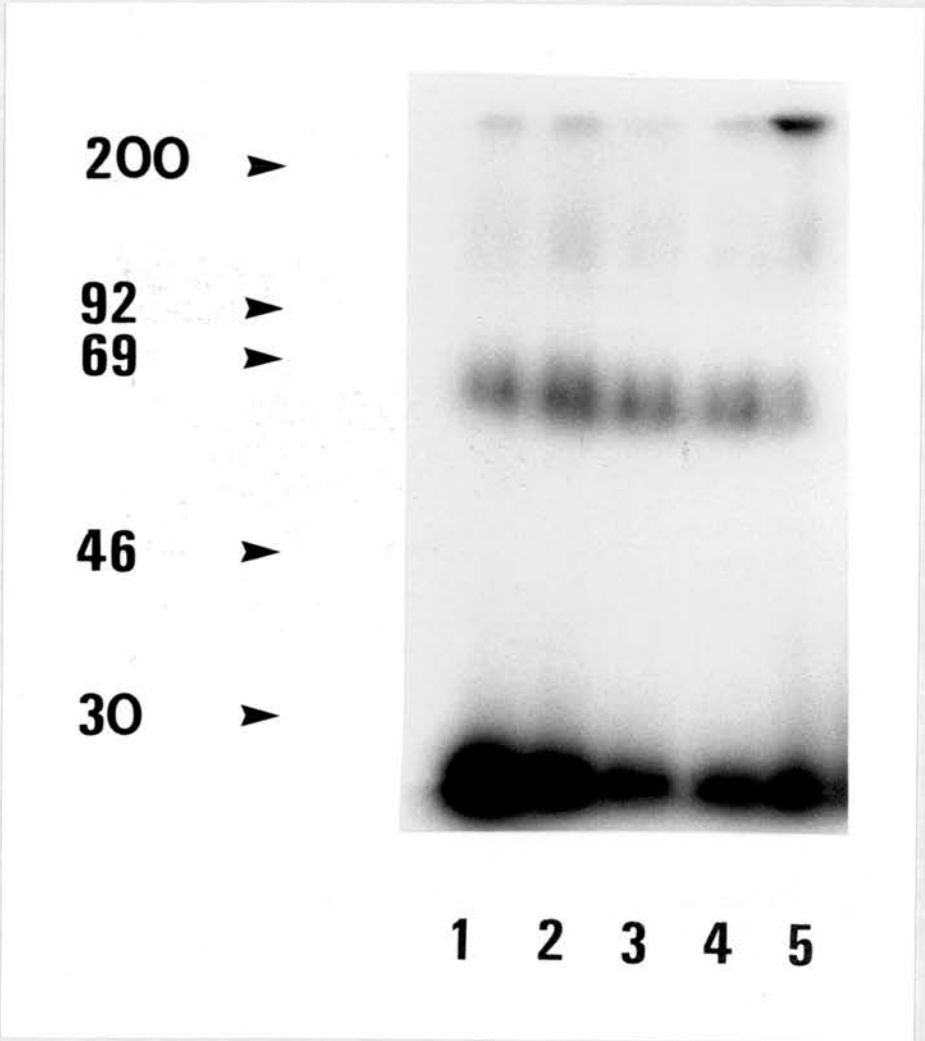

Figure 5.4.3. The temperature sensitivity of the IL-1 beta binding protein

Plasma samples were heated to 37° C, 40° C, 50° C, 60° C and 70° C (lanes 1-5) before incubation with IL-1 beta, and cross-linking. Molecular weight markers in kDa are shown.

binding following heating at 70° C.

5.4.c. N-Glycanase treatment

The partially purified binding protein prepared by ion exchange, was incubated with labelled IL-1 beta and the complex cross-linked. This was treated with N-Glycosidase F as described (Section 2.5.e.). The treated sample was then analysed by SDS-PAGE (Figure 5.4.4.). Treatment with N-Glycanase resulted in the appearance of a band migrating at about 47 kDa, suggesting that the unglycosylated protein has a molecular weight of around 30 kDa.

5.5. DISCUSSION

The results demonstrate the presence in both plasma and synovial fluid of a specific IL-1 beta binding protein. Interleukin 1 alpha is unable to bind or compete for sites of IL-1 beta binding to this protein. The protein associates with IL-1 beta to give a complex of molecular weight of approximately 100 kDa by gel filtration and 60 kDa by SDS-PAGE. The protein shows specific binding to a wheat germ agglutinin column, and following deglycosylation the molecular weight of the cross-linked complex is reduced to 47 kDa. The data suggest the presence of a 43 kDa binding factor, that following deglycosylation exhibits a molecular weight of 30 kDa. The binding protein appears to associate with other factor(s) under physiological conditions.

The protein has been partially purified from pooled synovial fluid samples. An initial ion-exchange separation step was followed by affinity chromatography on an IL-1 beta thiol Sepharose column. The binding protein could then be identified as a peak on reverse phase HPLC. Characterisation of the binding protein showed that association with IL-1 beta was slow at 4° C but rapid binding could be demonstrated at room temperature. The molecule was insensitive to heat inactivation, showing only slight loss of binding activity at 70° C.

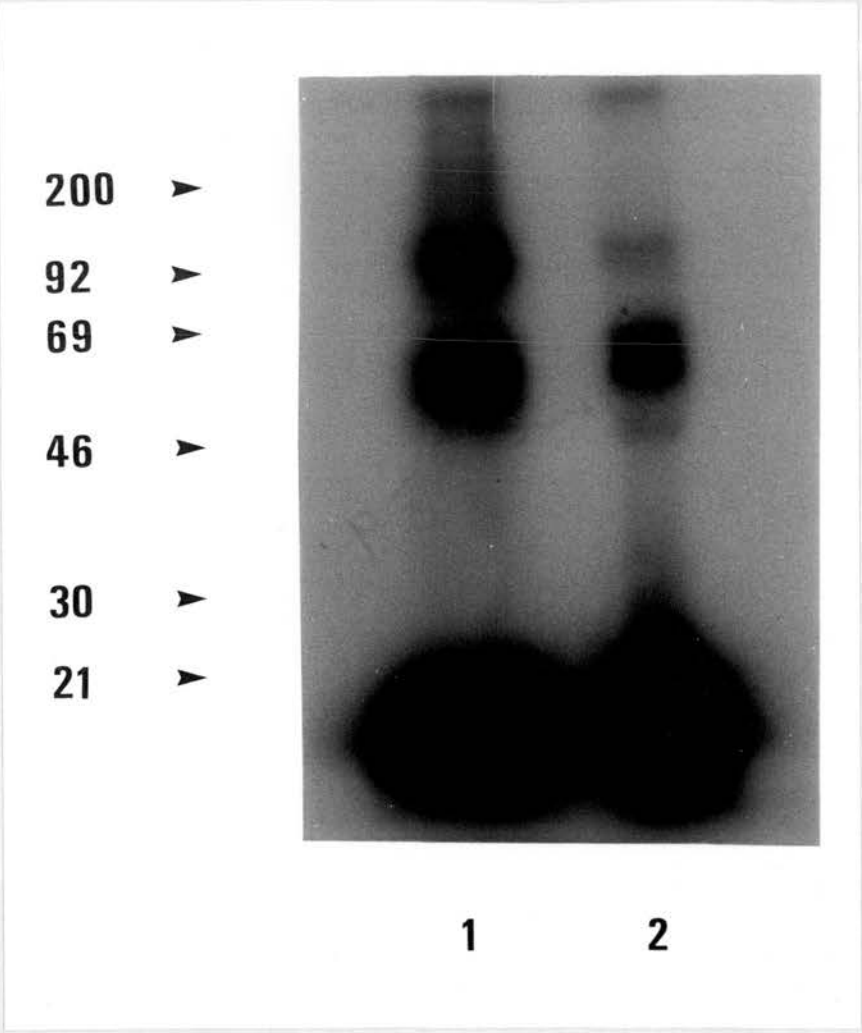

Figure 5.4.4. The effect of N-Glycanase on migration of the binding protein

Partially purified binding protein was incubated with labelled IL-1 beta and cross-linked. The complex was then treated with N-Glycosidase F and the molecular weight analysed by SDS-PAGE. Lanes 1 and 2 show untreated and deglycosylated binding protein respectively. Molecular weight markers in kDa are shown.

Gel filtration analysis of proteins that associate with iodinated IL-1 identified a high molecular weight species common for both IL-1 forms. This binding activity was not detected in unextracted plasma, but is revealed following the described chloroform treatment. It is possible that the high molecular weight factor may represent alpha $_2$ macroglobulin. It has been suggested that the binding of TGF beta to alpha $_2$M may occur through both covalent and non-covalent linkages (O' Conner-McCourt and Wakefield, 1987), and that some is readily removed by either acid or urea treatment (Huang *et al*, 1988). It is possible that chloroform denaturation results in removal of some molecules associated with alpha $_2$M, in this way uncovering sites suitable for IL-1 binding.

The second factor identified by gel filtration of plasma was able to bind only IL-1 beta resulting in a 100 kDa complex. The specificity of this association was tested by addition of 100 fold excess of unlabelled cytokine in the reaction. Using plasma, cold IL-1 alpha showed no competition of binding, but beta was able to reduce the size of the 100 kDa peak, this competition however was not completely effective. When synovial fluid was used as the source of binding molecule, excess unlabelled IL-1 beta removed all iodinated IL-1 beta binding at 100 kDa. It is therefore possible that the large amounts of alpha $_2$M present in serum and plasma are able to bind much of the cold cytokine thus reducing the pool available to compete for specific sites on other proteins.

Using cross-linking and SDS-PAGE analysis of plasma an IL-1 beta/binding protein complex was demonstrated at 60 kDa suggesting the presence of a 43 kDa factor. Specificity of association could be demonstrated. A comparable molecule was also identified in synovial fluid samples. When the pooled gel filtration peak was analysed by SDS-PAGE the complex migrated at 60 kDa, indicating that in physiological situations the 43 kDa binding protein associates with another factor(s) resulting in the larger iodinated peak seen by gel filtration. The inclusion of a reducing agent in the sample did not appear to alter the size of the band. However using affinity-purified material, reduction increased the size of the complex to about 70 kDa (J A

Symons, personal communication), suggesting that internal disulphide bonding was involved in maintaining the conformation of the molecule. A second cross-linking reagent was used to confirm that bands seen were not artefacts due to the system used.

The binding protein was partially characterised with regard to the binding kinetics and heat sensitivity. The rate of association with labelled IL-1 beta was slow at 4° C but at room temperature significant binding was demonstrable within an hour. The binding protein exhibited little temperature sensitivity even up to 70° C.

A recent report supports this finding of the association of iodinated IL-1 beta but not alpha with a high molecular weight binding protein (>66 kDa). A very slow rate of association of the factor with IL-1 beta at 4° C was also shown (Capper *et al*, 1990).

The presence of a band of broad molecular weight suggested that the factor may be glycosylated and we therefore used a lectin affinity column as an initial purification technique. Binding and specific elution of the protein from the wheat germ affinity column indicates that the molecule is glycosylated with carbohydrate containing N-acetyl glucosamine. Treatment of a partially-purified preparation of binding protein with N-Glycosidase F results in a complex with IL-1 beta that migrates at about 47 kDa. This indicates that the molecular weight of the unglycosylated molecule is approximately 30 kDa.

To enable large scale purification of the binding protein from pooled synovial fluid samples, ion exchange chromatography was used as an initial fractionation step. Binding protein eluted as two peaks at 0.15 and 0.25M sodium chloride, this may indicate differential glycosylation of the two forms.

The binding activity from the ion exchange purification was concentrated and further purified on an IL-1 beta affinity column. The column was prepared using an IL-1 beta mutein, with a cysteine replacing the lysine at residue 138. The characteristics of this IL-1 beta form have been studied (Wingfield *et al*, 1989). The molecule shows similar conformation to the native form, as

assessed by NMR, and has comparable receptor binding affinity and biological activity. Binding protein eluted from the column with pH 3 glycine, and showed similar specificity to the unpurified molecule.

HPLC analysis of the preparation indicated the presence of multiple protein peaks, one of the major ones demonstrating high levels of IL-1 beta binding activity.

There are a number of reports demonstrating the release of receptor molecules *in vivo*, resulting in soluble, high-affinity, ligand binding moieties. As discussed, various soluble cytokine receptors have been described, many of which show an inhibitory effect on the bioactivity of the ligand by preventing receptor binding. It is possible that the IL-1 beta binding protein described here is a soluble IL-1 receptor molecule.

Further studies concerning the cellular source of the binding protein have been carried out, they indicate that it is produced by PHA stimulated peripheral blood mononuclear cells (Symons *et al*, 1990), and the Raji B cell line (Symons and Duff, 1990). From cell surface cross-linking experiments the latter study indicates that the molecule may be a solubilised form of the Raji IL-1 receptor that is released following cleavage by a cell surface protease.

Studies on the IL-1 receptor have suggested that two distinct forms exist, the 80 kDa molecule found predominantly on T cells and fibroblasts and the 60 kDa form characterised on B cells (Chizzonite *et al*, 1989). The 60 kDa molecule shows preferential binding to IL-1 beta (Scapigliati *et al*, 1989). Recent evidence, however, suggests a more complicated model in which many B cell lines possess two receptor forms. The Raji line appears to be uncommon in having only the smaller molecule (Benjamin and Dower, 1990; Symons and Duff, 1990).

The cloned 80 kDa (type 1) IL-1 receptor has been expressed as a truncated 62 kDa molecule and shown to retain IL-1 alpha binding activity (Dower *et al*, 1989). This soluble receptor has IL-1 inhibitory activity in both *in vivo* and *in vitro* assays (Maliszewski *et al*, 1990; Fanslow *et al*, 1990). However no comparable natural molecule has yet been described in

biological fluids.

The preferential IL-1 beta binding characteristics of the Raji IL-1 (type 2) receptor adds further evidence that the soluble binding protein described in the present work may be a soluble form of this cell-surface IL-1 receptor.

The association of IL-1 beta with the synovial fluid binding protein has been shown by Scatchard analysis to be of comparable affinity to that with the 80 kDa receptor on EL4 cells, suggesting a potential ability to compete for IL-1 binding with the cell surface receptor (Symons *et al*, 1990). Recent studies show that the affinity purified protein from Raji cell supernatants inhibits the binding of iodinated IL-1 beta to the receptor on EL4 and Raji cells, but does not affect IL-1 alpha binding (J A Symons, personal communication). The binding protein may, therefore, act as a significant physiological regulator of IL-1 beta activity *in vivo*.

6. RESULTS: Interleukin 1 beta processing

6.1. INTRODUCTION

Cleavage of the inactive IL-1 beta precursor to the biologically active mature molecule is a potential site for the pharmacological modulation of IL-1 activity. As the IL-1 alpha precursor exhibits receptor binding and is active biologically, inhibition of its processing to the mature form is unlikely to have a major effect on the overall biological response. Interleukin 1 alpha is also thought predominantly to be a cell associated molecule, therefore inhibition of its activity or production may have less impact *in vivo* than alterations in bioactive IL-1 beta levels.

The IL-1 beta processing event is poorly characterised, it has been suggested that a cell surface or secreted enzyme is responsible for proteolytic cleavage, as mature IL-1 beta is rarely found intracellularly (Black *et al*, 1988). However attempts to isolate the processing enzyme have also identified factors in cytosolic (Black *et al*, 1989b; Kostura *et al*, 1989) and membrane preparations (Black *et al*, 1988). The cytosolic processing activities described are shown to result in the correct cleavage product as determined by N-terminal sequencing, and to increase the bioactivity of the IL-1 beta preparation.

The importance of processing at the correct site, Asp^{116}-Ala^{117}, has been shown by Black *et al* (1988) who used a variety of well characterised enzymes to cleave the IL-1 beta propeptide. This showed that enzymic activities resulting in slightly larger mature IL-1 molecules, even elongation by only a few amino acids at the N-terminus, resulted in a considerable loss of bioactivity. This led to the suggestion that the factor responsible for production of the optimal mature IL-1 beta molecule was a specific enzyme with an unusual substrate specificity.

A recent report however has indicated that *in vivo*, particularly in the inflammatory, state another mechanism may operate. Hazuda *et al* (1990) showed that enzymes present in inflammatory exudate fluids cleave recombinant pro IL-1 beta to a biologically active form. The specificity of this processing reaction was not determined, but it was demonstrated that enzymes such as elastase, collagenase and cathepsin G are all able to increase the level of

lower molecular weight IL-1 products. It is therefore postulated that precursor IL-1 beta may be released either as a result of specific secretion or cellular damage, at the site of an inflammatory reaction. This could then be cleaved by proteolytic enzymes derived from inflammatory leukocytes to give increased local IL-1 bioactivity.

The aim of the present study was to identify factors capable of processing the IL-1 beta propeptide, and then to attempt to inhibit the reaction with either common protease inhibitors or synthetic peptides constructed from sequences around the cleavage site of the precursor molecule as shown in Figure 6.1.1..

6.2. CELLULAR PROCESSING OF IL-1 BETA

6.2.a. Sources of IL-1 beta propeptide and processing activity

To determine a suitable source of IL-1 beta propeptide and of processing enzyme activity various monocytic cell lines were stimulated as described (Section 2.1.e.). The supernatants and cell lysates were separated by SDS-PAGE followed by Western blotting (Sections 2.9.a.), and immunoreactive IL-1 beta visualised using the avidin biotin detection system (2.9.c.). Figure 6.2.1. shows IL-1 levels in cell lysates and supernatants of THP-1, HL-60 and U937 cells, production by stimulated and unstimulated cells are shown. The production of 31 kDa propeptide in cell lysates was enhanced by PMA and zymosan in each cell line tested. The HL-60 and U937 lines showed minimal propeptide levels when unstimulated, and LPS did not significantly induce production. In culture supernatants propeptide was found in media from THP-1 and U937 cells cultured with PMA. However mature IL-1 beta, co-migrating with the recombinant 17 kDa form, was only detected in THP-1 supernatants.

6.2.b. Inhibition of processing in whole cell culture

THP-1 cells were cultured with PMA to stimulate IL-1 beta production. Protease inhibitors were also added in an attempt to inhibit processing of the precursor molecule (Section 2.1.f.). Figure 6.2.2. shows Western blots of

Met Ala Glu Val Pro Glu Leu Ala Ser Glu Met Met Ala Tyr Tyr Ser Gly Asn Glu
19
Asp Asp Leu Leu Phe Glu Ala Asp Gly Pro Lys Gln Met Lys Cys Ser Phe Gln Asp
38
Leu Asp Leu Cys Pro Leu Asp Gly Gly Ile Gln Leu Arg Ile Ser Asp His His Tyr
57
Ser Lys Gly Phe Arg Gln Ala Ala Ser Val Val Val Ala Met Asp Lys Leu Arg Lys
76
Met Leu Val Pro Cys Pro Gln Thr Phe Gln Glu Asn Asp Leu Ser Thr Phe Phe Pro
95
Phe Ile Phe Glu Glu Glu Pro Ile Phe Phe Asp Thr Trp Asp Asn Glu Ala Tyr Val
114
His Asp Ala Pro Val Arg Ser Leu Asn Cys Thr Leu Arg Asp Ser Gln Gln Lys Ser
133
Leu Val Met Ser Gly Pro Tyr Glu Leu Lys Ala Leu His Leu Gln Gly Gln Asp Met
152
Glu Gln Gln Val Val Phe Ser Met Ser Phe Val Gln Gly Glu Glu Ser Asn Asp Lys
171
Ile Pro Val Ala Leu Gly Leu Lys Glu Lys Asn Leu Tyr Leu Ser Cys Val Leu Lys
190
Asp Asp Lys Pro Thr Leu Gln Leu Glu Ser Val Asp Pro Lys Asn Tyr Pro Lys Lys
109
Lys Met Glu Lys Arg Phe Val Phe Asn Lys Ile Glu Ile Asn Asn Lys Leu Glu Phe
128
Glu Ser Ala Gln Phe Pro Asn Trp Tyr Ile Ser Thr Ser Gln Ala Glu Asn Met Pro
147
Val Phe Leu Gly Gly Thr Lys Gly Gly Gln Asp Ile Thr Asp Phe Thr Met Gln Phe
166
Val Ser Ser
269

Taken from Auron *et al*, 1984.

Peptide a, amino acids 102-138 MWt= 4.3 kDa ———
Peptide b, amino acids 111-122 MWt= 1.36 kDa – – – –
Peptide c, amino acids 101-112 MWt= 1.48 kDa - - - - - - -
Arrow represents the IL-1 beta propeptide cleavage site

Figure 6.1.1. The amino acid sequence of the IL-1 beta precursor

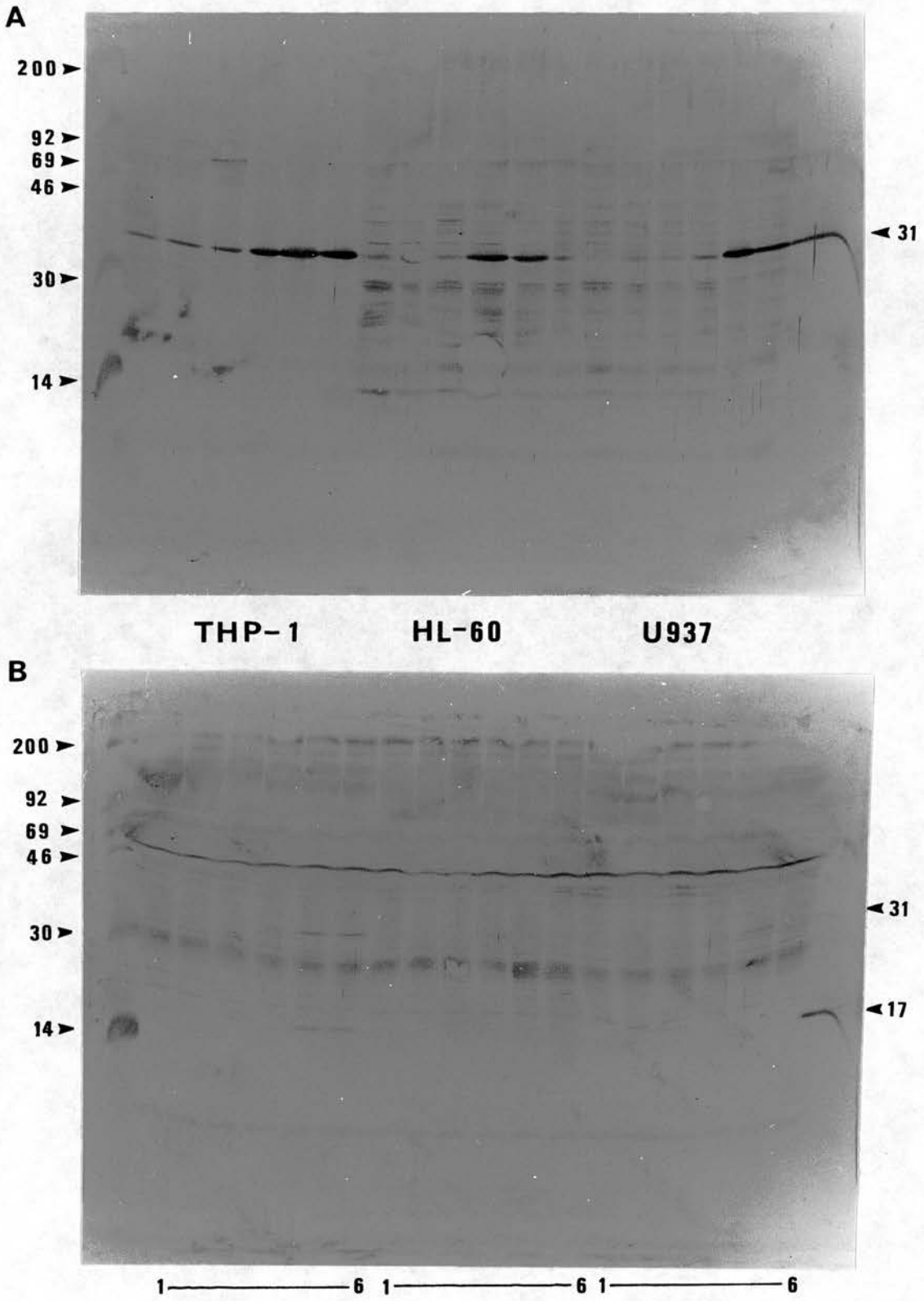

Figure 6.2.1. Production and processing of IL-1 beta by whole cells

Production of IL-1 beta in lysates (A) and culture supernatants (B) of THP-1, HL-60 and U937 cells. Cells were cultured alone (lane 1), with 100ng/ml or 10ug/ml LPS (2 and 3), 1mg/ml zymosan (4) and 0.1 or 1ug/ml PMA (5 and 6). The last lane in panel B shows recombinant IL-1 beta. Molecular weight markers in kDa are indicated.

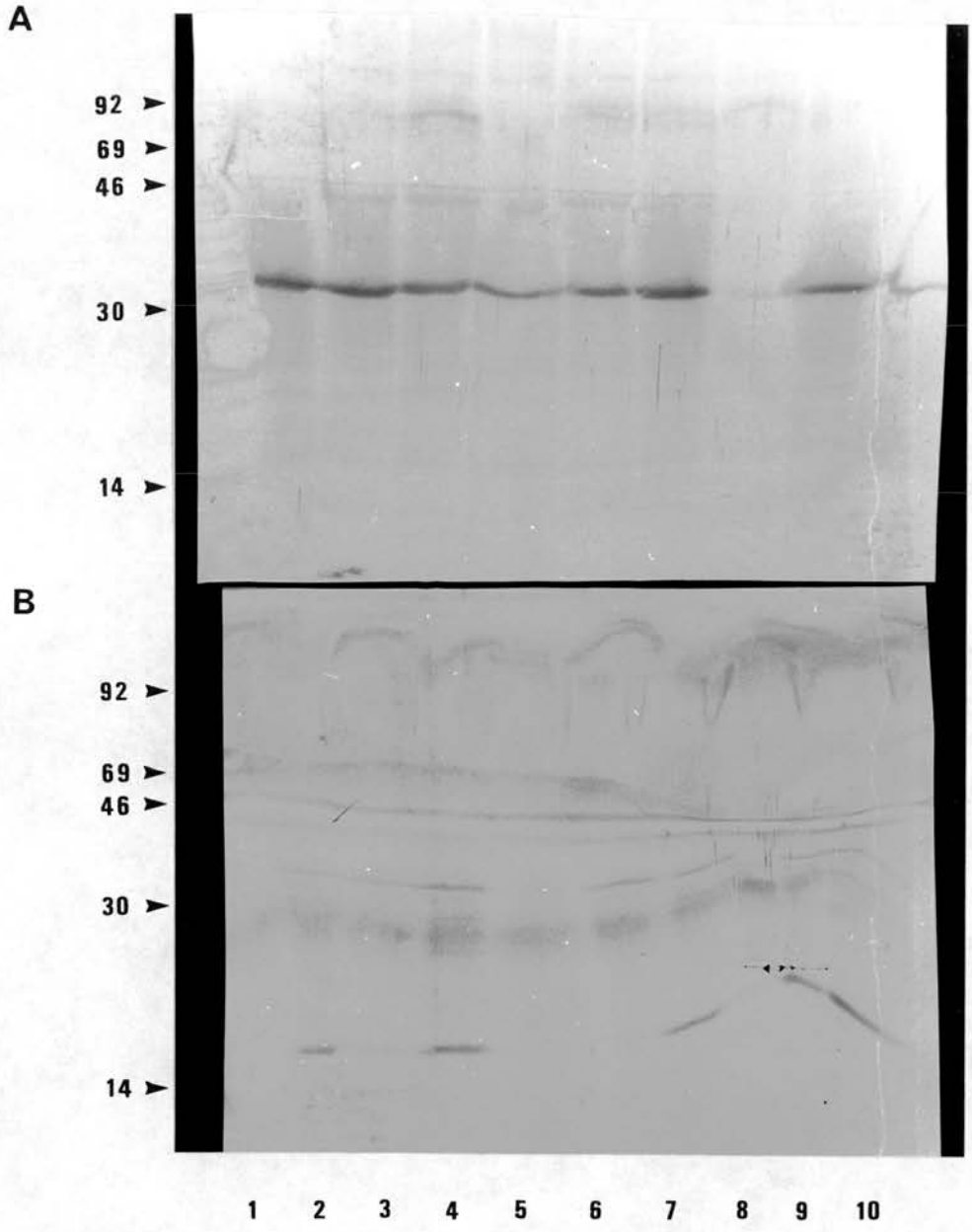

Figure 6.2.2. Inhibition of cellular IL-1 beta processing in whole cells

Detection of IL-1 beta in lysates (A) and culture supernatants (B) by Western blotting. THP-1 cells were cultured in media alone (lane 1), stimulated with 100ng/ml PMA (2), or PMA with; aprotinin (3), pepstatin (4), PMSF (5), leupeptin (6), 100ng/ml 3,4 DC (7), 100ng/ml 1,10 PA (8), 10ng/ml 3,4 DC (9) and 10ng/ml 1,10 PA (10). The position of molecular weight markers in kDa are indicated.

the cell lysates (A) and culture supernatants (B) to determine the production and size of IL-1 beta protein. The figure shows that similar levels of the precursor are produced in all cultures apart from that containing 1,10 phenanthroline (1,10-PA, 100ng/ml; lane 8). Cells cultured with this concentration of 1,10-PA exhibited reduced viability by trypan blue exclusion. Lower concentrations did not affect the amount of propeptide in the cell lysate (lane 10). In culture supernatants aprotinin (lane 3) and leupeptin (lane 6) reduced the appearance of mature IL-1 beta but not the propeptide indicating inhibition of enzyme processing. PMSF reduced the levels of both molecules (lane 5). The other protease inhibitors had no effect on the level of processed IL-1 beta.

6.2.c. The effect of protease inhibitors on IL-1 beta mRNA levels

The IL-1 beta mRNA levels in THP-1 cells following culture with protease inhibitors were determined by slot blot analysis (Section 2.8.b.) and are shown in Figure 6.2.3.. Low levels of IL-1 beta mRNA (A) were seen in unstimulated cells (lane 1), but levels were raised following culture with PMA (lane 2). Aprotinin, pepstatin or 3,4 dichloroisocoumarin did not alter IL-1 beta mRNA levels. However PMSF, leupeptin and 1,10-PA considerably reduced IL-1 beta mRNA compared to stimulated cells. To ensure that the results did not reflect differences in sample loading between wells, the filter was stripped and reprobed with 7B6 cDNA, which encodes a cell cycle-independent message. When hybridised with 7B6 (6.2.3.B), sample loading was shown to be comparable between wells.

The effect of protease inhibitors on pro IL-1 beta processing and mRNA levels in the whole cell system is summarised in Table 6.2.1..

6.3. PROCESSING OF CELL LYSATE (NATURAL) PRO IL-1 BETA

Stimulated THP-1 cell lysate was prepared as a source of 31 kDa IL-1 beta as described in Section 2.2.a.. The ability of a membrane preparation, whole cells and the cell lysate itself to effect processing were examined over a 1-24 hour time period. The inhibitory activity of both aprotinin and

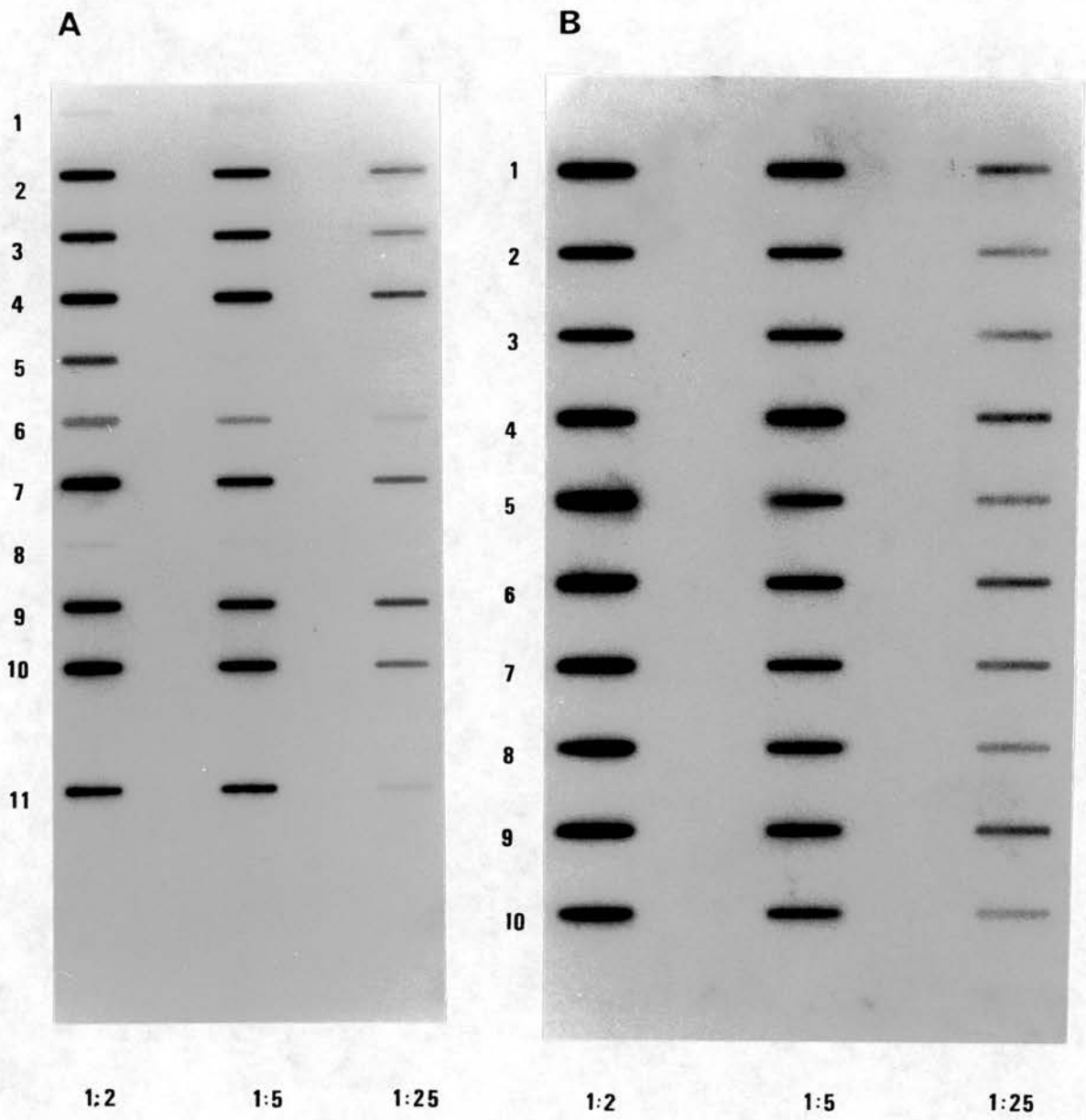

Figure 6.2.3. mRNA levels in cells cultured with protease inhibitors

Slot blots were probed with cDNA coding for IL-1 beta (A) or 7B6 (B) mRNA. THP-1 cells were cultured in media alone (lane 1), stimulated with PMA (2), or PMA with; aprotinin (3), pepstatin (4), PMSF (5), leupeptin (6), 100ng/ml 3,4 DC (7), 100 ng/ml 1,10 PA (8), 10ng/ml 3,4 DC (9), or 10ng/ml 1,10 PA (10). Lane 11 shows IL-1 beta cDNA as a positive control. Dilutions of 1:2, 1:5 and 1:25 of the mRNA preparations are shown.

Protease inhibitor	Extracellular IL-1 beta levels		
	Pro IL-1 beta	17 kDa IL-1 beta	IL-1 beta mRNA
Aprotinin	–	▼	–
Pepstatin	–	–	–
Phenyl methyl sulphonyl fluoride	▼	▼	▼
Leupeptin	–	▼	▼
3,4 dichloro-isocoumarin	–	–	–
1,10 phenanthroline	–	–	–

Table 6.2.1. The effect of protease inhibitors on the cellular processing of IL-1 beta

the synthetic 37 amino acid peptide (Peptide a), shown in Figure 6.1.1., was tested.

The lysate preparation (Figure 6.3.1.A) showed IL-1 beta processing ability at late time points (12 and 24 hours; lanes 1a-5a), but these bands were not seen when aprotinin (lanes 1b-5b) or synthetic peptide (lanes 1c-5c) were present in the reaction. Addition of membrane preparations resulted in a faint band co-migrating with recombinant IL-1 beta at the 6 hour time point (Figure 6.3.1.B), no comparable bands were seen in the reaction with aprotinin or peptide added. When fresh whole cells were used as processing activity more distinct 17 kDa bands were seen particularly at 6 and 12 hours (Figure 6.3.1.C). Again no bands were seen if aprotinin or peptide was added.

6.4. PROCESSING OF RECOMBINANT PRO IL-1 BETA

6.4.a. Cellular localisation of processing activity

To make a preliminary assessment of processing activity, cell lysate and membrane preparations were tested for their ability to cleave recombinant pro IL-1 beta (Section 2.2.b.). Two different monocytic lines were tested and the effect on processing of heating to 50° C, to destroy enzyme activity, was also determined (Figure 6.4.1.). The figure shows that recombinant IL-1 beta propeptide migrates above the 30 kDa marker. It undergoes little degradation during the course of the incubation (lane 10), the only smaller bands in the preparation migrate slightly faster than the recombinant propeptide. The addition of membranes from either THP-1 or HL-60 (lanes 5 and 3 respectively) cells resulted in the appearance of a number of bands at >17 kDa, and a band that co-migrated with recombinant mature IL-1 beta. Cell lysates showed some processing activity (lanes 2 and 4). Stimulation of the cells with PMA did not increase the intensity of the processed ~17 kDa band (lanes 6 and 7), heating of the membrane and lysate preparations reduced the level of the 17 kDa form (lanes 8 and 9).

The processing ability of THP-1 cell lysates, membrane preparations and culture supernatant was assessed with and without prior stimulation by LPS or PMA (Section 2.2.a.). Figure 6.4.2. demonstrates that none of the

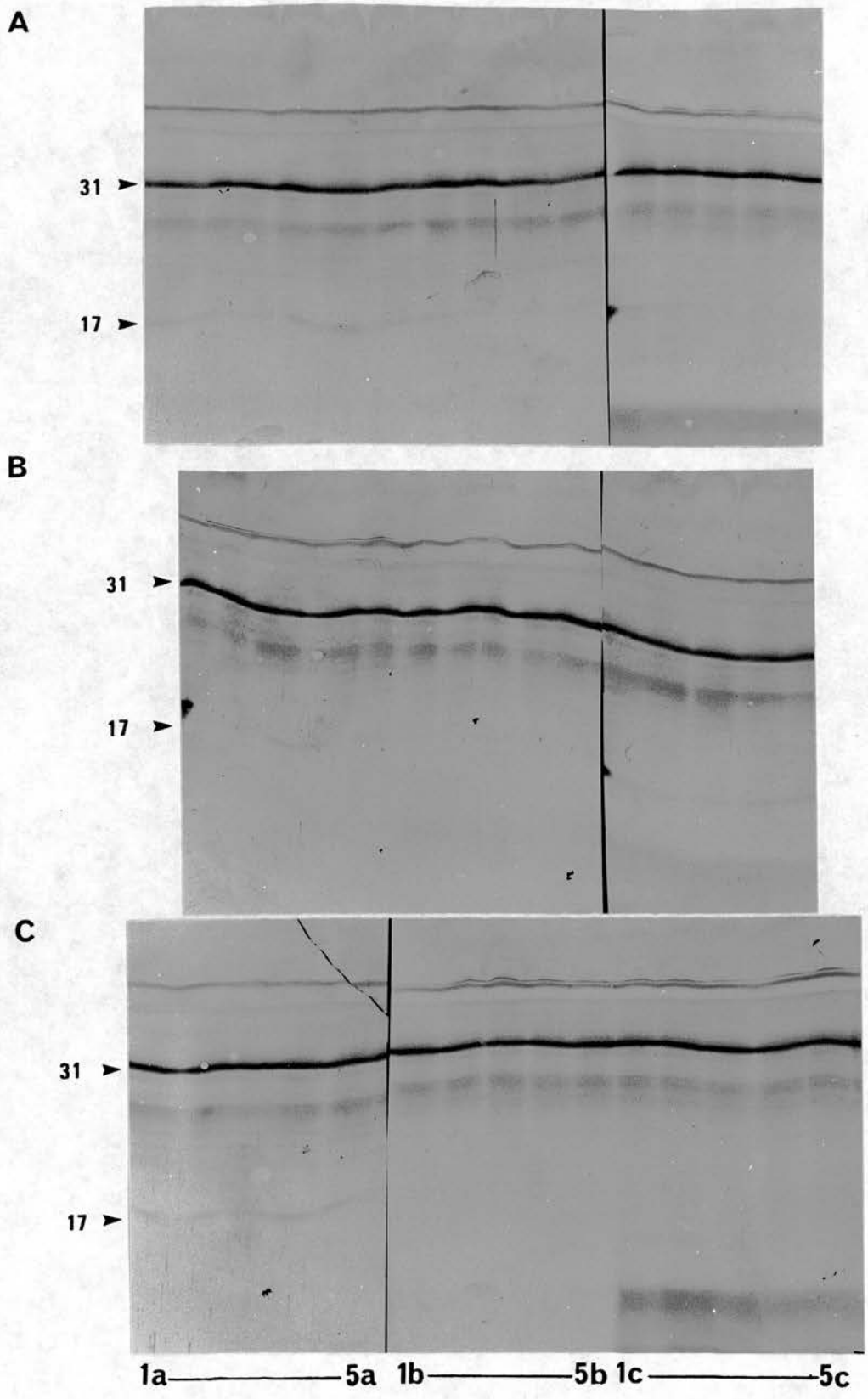

Figure 6.3.1. Processing of IL-1 beta in cell lysates

THP-1 cell lysates were used as pro IL-1 beta source. Lysates were incubated alone (A), with membrane preparation (B) or fresh cells (C). The reaction was incubated for 1, 2, 6, 12, and 24 hours (lanes 1-5). Aprotinin 0.67U/ml (b) or 10^{-5}M peptide a (c) were added to the reaction.

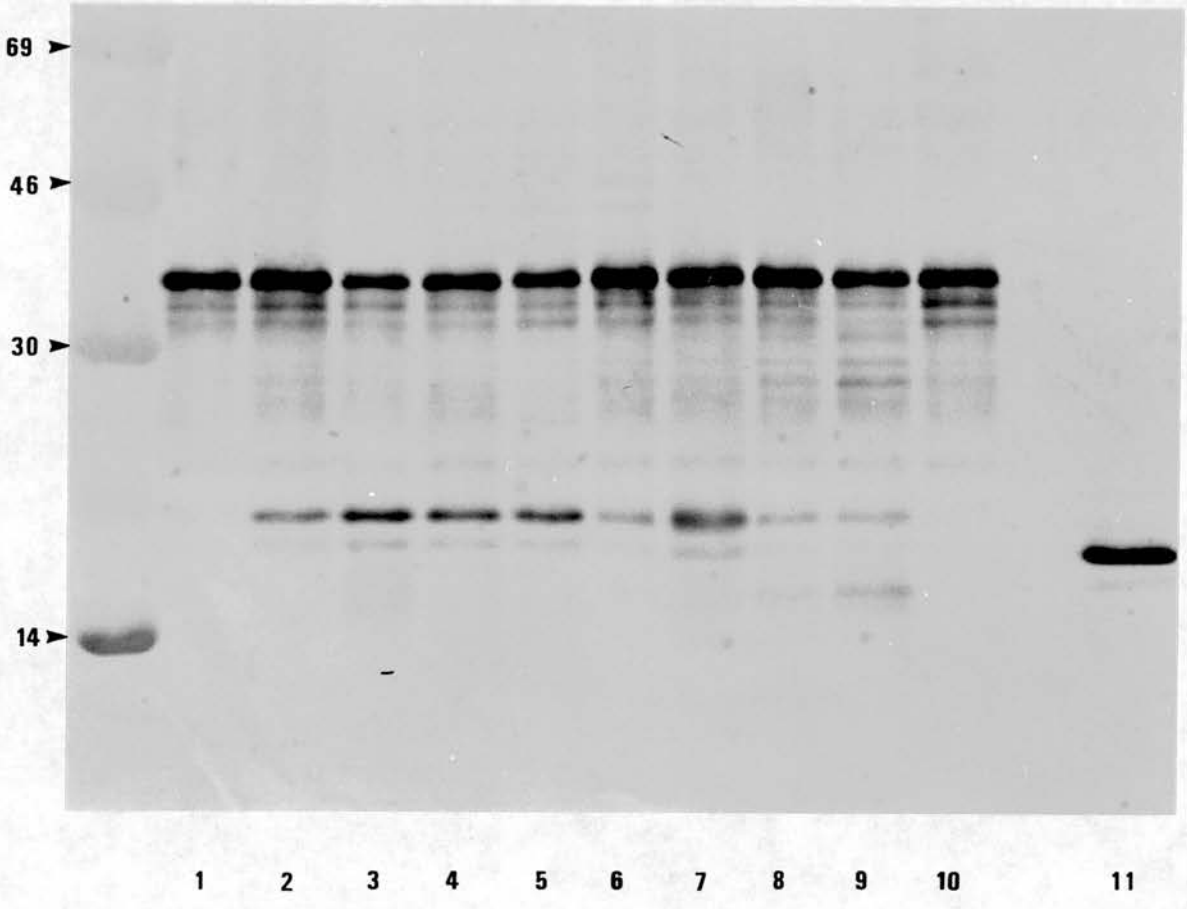

Figure 6.4.1. Processing of recombinant pro IL-1 beta

Recombinant pro IL 1 beta was incubated for 2 hours at 37° C with various cell lysate and membrane preparations. Lane 1 shows propeptide alone without incubation, lanes 2 and 3 propeptide incubated with HL-60 lysate and membranes respectively, lanes 4 and 5 THP-1 cell lysates and membranes, lanes 6 and 7 PMA stimulated THP-1 cell lysates and membranes, lanes 8 and 9 heat treated THP-1 lysates and membranes and lane 10 propeptide alone incubated for 2 hours at 37° C. Lane 11 shows recombinant 17 kDa IL-1 beta. Molecular weight markers in kDa are shown.

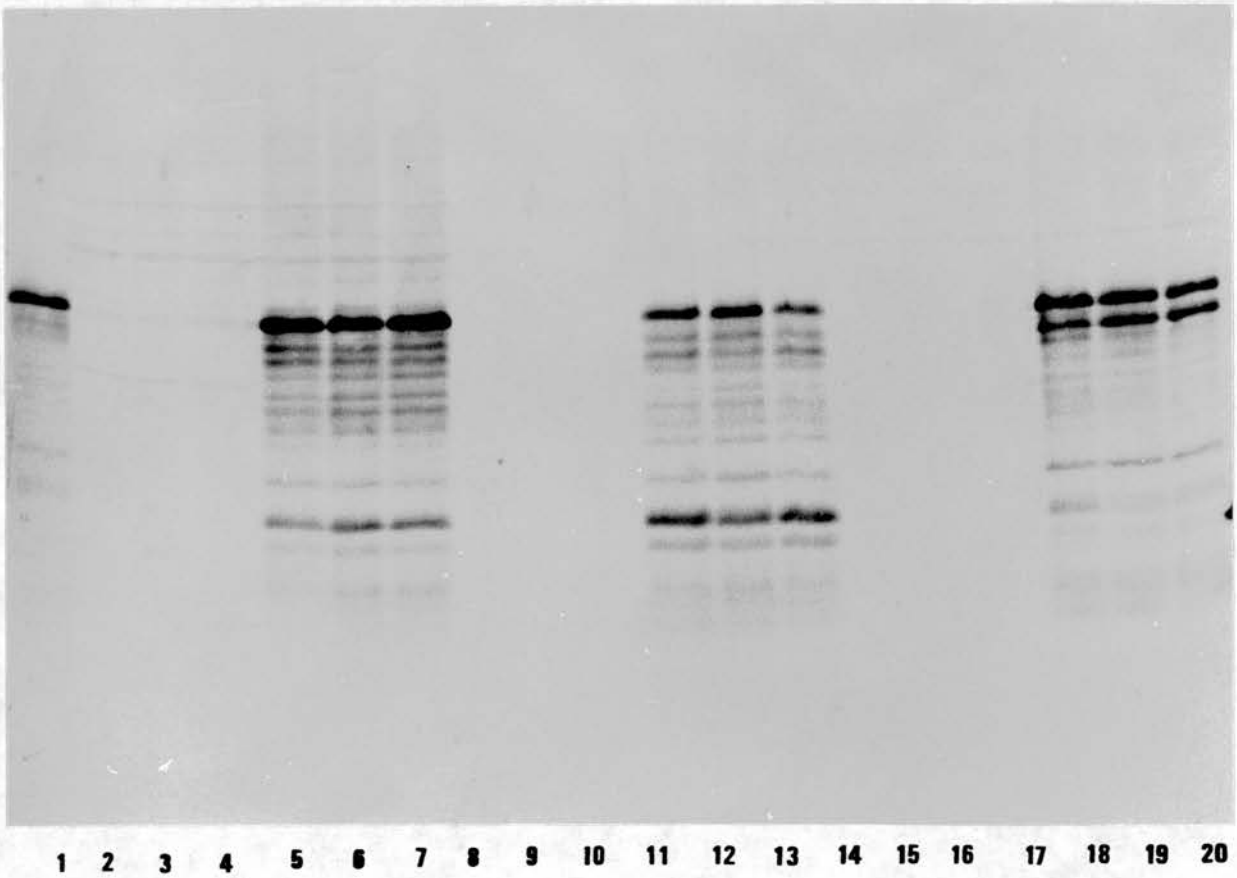

Figure 6.4.2. The processing ability of cell lysates, membranes and culture supernatants

The ability of THP-1 cell lysates (lanes 2-7), membrane preparations (lanes 8-13) and culture supernatants (lanes 14-19) to process the IL-1 precursor. Lane 1 shows recombinant pro IL-1 beta alone and lanes 2-4, 8-10 and 14-16 show lysates, membranes and supernatant alone. Lanes 2, 5, 8, 11, 14 and 17 represent unstimulated cells; 3, 6, 9, 12, 15 and 18 cells stimulated with 100ng/ml LPS; 4, 7, 10, 13, 16, 19, cells stimulated with 100ng/ml PMA. Lane 20 shows recombinant 17 kDa IL-1 beta.

preparations alone showed intrinsic IL-1 beta activity at 17 kDa, but that addition of cell lysates resulted in multiple bands of IL-1 immunoreactivity one of which co-migrated with the mature molecule. When membrane preparations were used as the source of processing activity many intermediate IL-1 forms were seen, the intensity of the precursor band was much lower than in other reactions and an increase in intensity of the ~17 kDa molecule is observed when compared to the reaction using cell lysates. Culture supernatants showed a different pattern of bands when compared to the other enzyme sources, a very faint band was seen co-migrating with the mature IL-1 beta molecule. Stimulation of the cells before fractionation did not appear to alter their processing ability.

6.4.b. Time course of production of processed IL-1 beta

The rate of production of the 17 kDa IL-1 form was determined by stopping the reaction at various time points up to five hours (Section 2.2.c). The results are shown in Figure 6.4.3.. The first panel (A) shows the degradative products of the incubation of the propeptide alone at 37° C, very little low molecular weight material was seen though an obvious decrease in the level of intensity of the 33 kDa band was observed. Incubation with THP-1 cell lysates (panel B) showed little processing in this experiment, though a faint band was seen at ~17 kDa at later time points. Panel C shows the effect of membranes in the processing reaction, the 17 kDa band was evident within 30 minutes and was most intense at 1.5-2 hours. After this time point there appeared to be some degradation of the products. From the time course it was not apparent whether the higher molecular weight bands were processing intermediates or products of other enzyme activities present in the membrane preparation.

6.4.c. Inhibition of the processing reaction

Peptides a, b and c (Section 2.2.e.) were tested for their ability to inhibit the processing reaction, using membrane preparations as a source of cleavage activity (Section 2.2.a). None of the peptides alone showed any IL-1

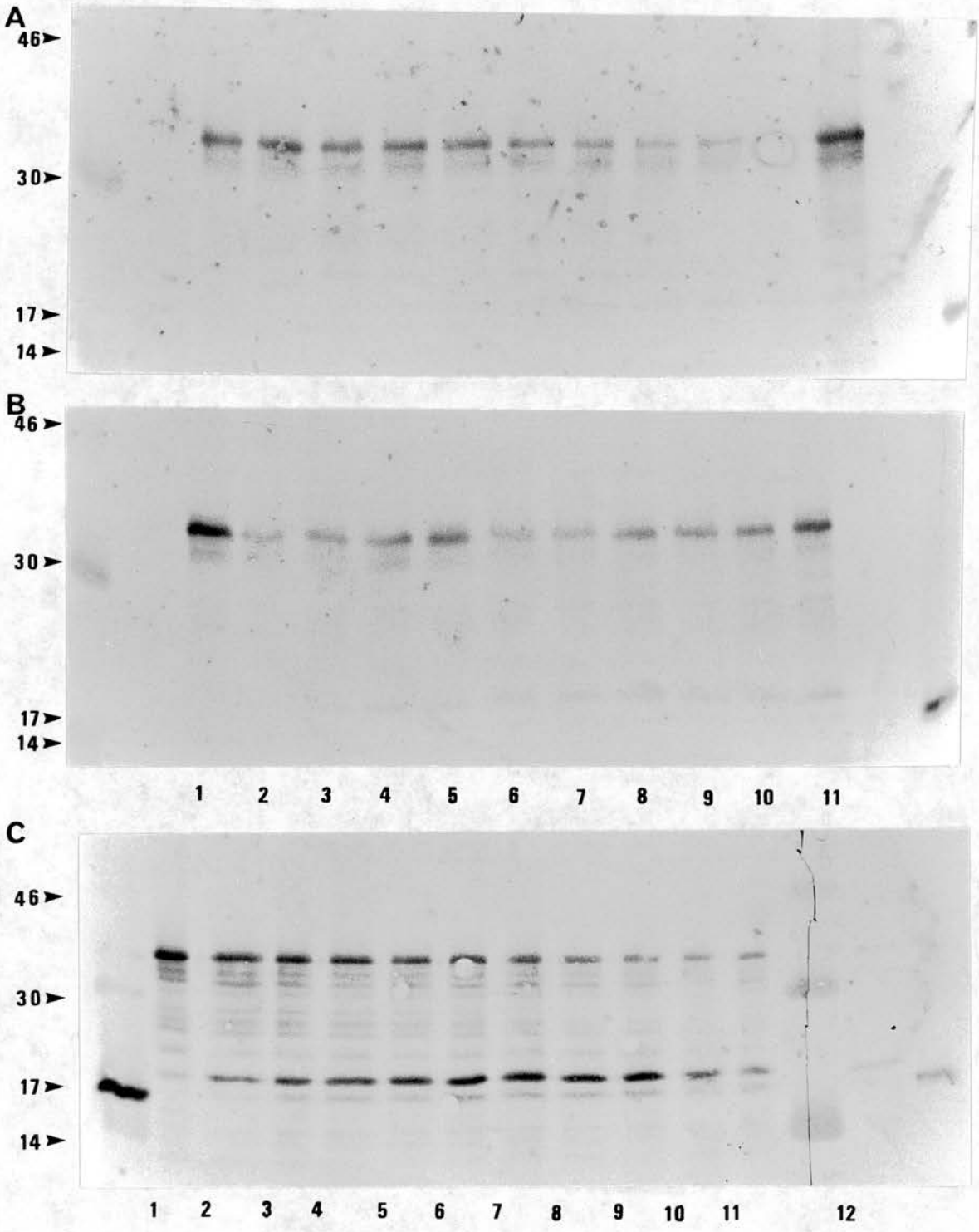

Figure 6.4.3. Time course of pro IL-1 beta processing

Recombinant IL-1 beta propeptide was incubated with tissue culture media (A), THP-1 cell lysate (B) or membrane preparation (C) Aliquots of incubation were taken at the start of reaction (lane 1), 15 mins (2), 30 mins (3), 45 mins (4), 1 hr (5), 1.5 hr (6), 2 hr (7), 2.5 hr (8), 3.0 hr (9), 4 hr (10), and 5 hr (11). Lane 12 on panel C shows the inhibition of antibody binding by including 100 fold excess of mature IL-1 beta in the primary antibody incubation. Molecular weight markers in kDa are shown.

beta immunoreactivity (lanes 13-22; Figure 6.4.4.). The peptides were not able to inhibit the appearance of the IL-1 band co-migrating with the mature form, b and c however did alter the intensity of higher molecular weight bands. Using this system aprotinin did not inhibit the production of the ~17 kDa molecule but did alter the banding pattern at higher molecular weights.

Protease inhibitors were tested for their ability to inhibit the processing of pro IL-1 beta (Section 2.2.d.). None of the protease inhibitors alone showed any cross-reacting bands by Western analysis (Figure 6.4.5.). In some cases protease inhibitors altered the intensity of certain IL-1 beta immunoreactive bands, but only 3,4 DC at 10ug/ml was able to prevent the appearance of the ~17 kDa molecule (lane 12).

The effect of elastatinal on processing is shown in Figure 6.4.6.. The inhibitor did not affect the appearance of any band at 1 or 10mM, whereas 3,4 DC again prevented processing to the ~17 kDa form.

6.5. BIOACTIVITY OF PROCESSED IL-1 BETA

The bioactivity produced by the processing reaction was determined by EL4/CTLL assay (Section 2.1.b.). The results are shown in Table 6.5.1.. Sample concentrations above 1% showed some cytotoxicity in the assay, the values presented are from preparations tested at 0.1%. Propeptide alone resulted in no detectable activity, but when spiked with the recombinant mature form showed >100% recovery of bioactivity. Processed IL-1 was readily detectable in the EL4 assay. From the amount of propeptide used in the initial reaction (275ng) it was estimated that about 34.5% had been converted to a bioactive form.

6.6. DISCUSSION

Stimulated THP-1 cells were shown to produce the IL-1 beta propeptide molecule intracellularly, the precursor could be detected extracellularly as could the mature 17 kDa molecule. The cellular processing event was prevented by co-culture with the protease inhibitor aprotinin, no effect on

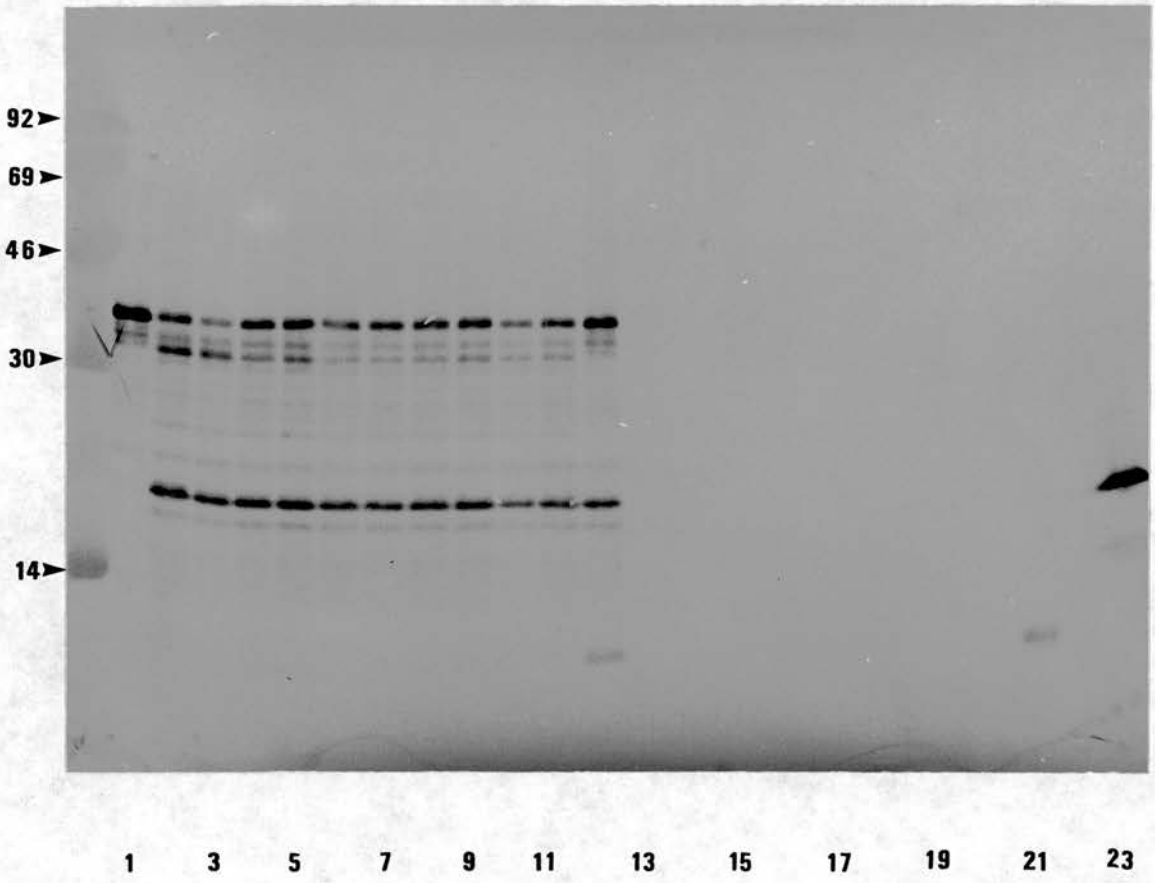

Figure 6.4.4. The effect of synthetic peptides on the processing reaction

The processing of pro IL-1 beta by THP-1 membranes alone (lane 2) or with 10^{-5}, 10^{-6} or 10^{-7}M peptide a (lanes 3-5), peptide b (lanes 6-8), peptide c (lanes 9-11) or 0.67 U/ml aprotinin (lane 12) is shown. Lanes 13-22 show the peptides alone. Lane 1 shows recombinant precursor IL-1 beta and lane 23 mature IL-1 beta.

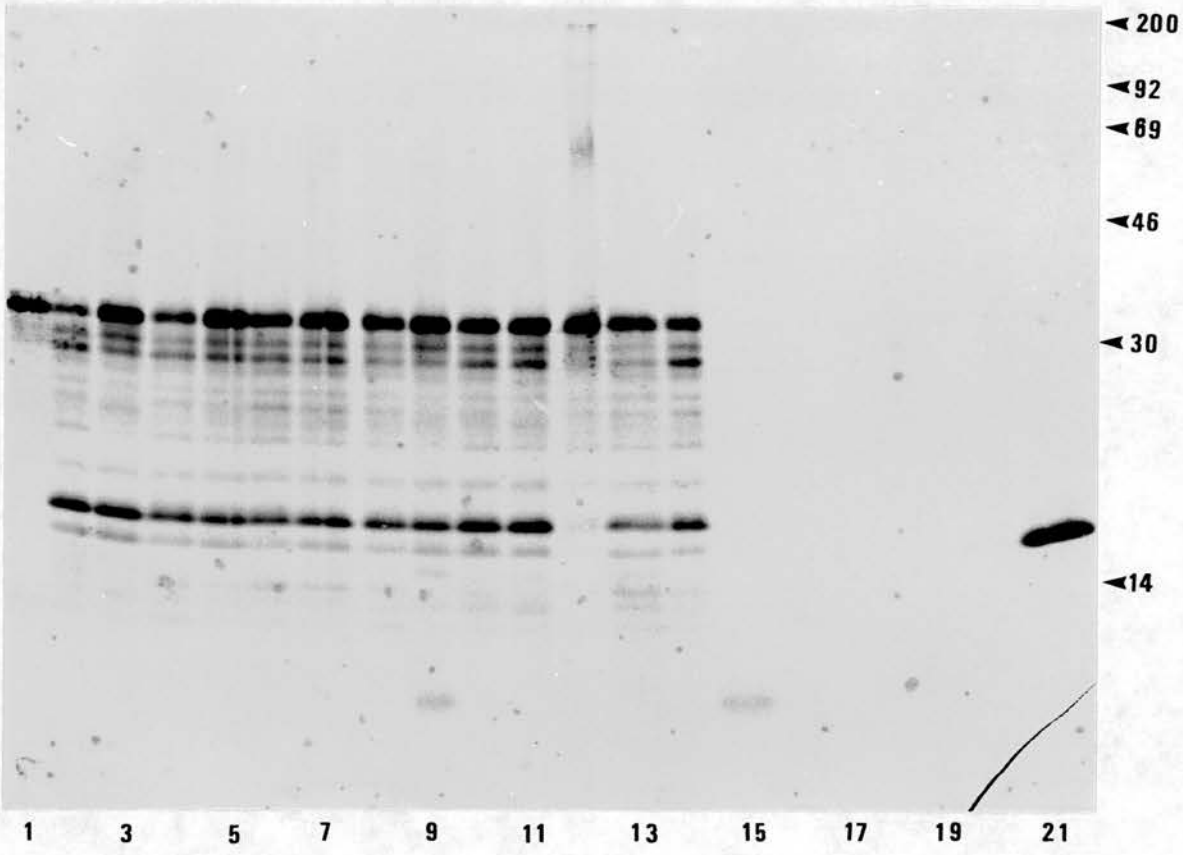

Figure 6.4.5. The effect of protease inhibitors on recombinant pro IL-1 beta processing

Recombinant pro IL-1 beta was incubated alone (lane 1), with THP-1 membranes (2), and with membranes plus 1x and 10x concentrations of protease inhibitors; aprotinin (3 and 9), pepstatin (4 and 10), leupeptin (5 and 11), 3,4 DC (6 and 12), 1,10 PA (7 and 13) and PMSF (8 and 14). Lanes 15-20 show 10x concentrations of protease inhibitors alone. Lane 21 shows recombinant mature IL-1 beta. Molecular weight markers in kDa are shown.

Figure 6.4.6. The effect of elastatinal on IL-1 beta processing

Recombinant pro IL-1 beta was incubated alone (lane 1), with THP-1 membranes (2), and membranes with 1x and 10x elastatinal (3 and 4) or 3,4 DC (5). Lane 6 shows recombinant 17 kDa IL-1 beta.

Sample	IL-1 bioactivity pg/ml	
	1:1000 dilution	Recovery
Propeptide alone	<0.1pg/ml	-
Propeptide + 1ng/ml mature IL 1	3pg/ml	-
Propeptide + membrane preparation	95pg/ml	95ng/ml 34.5%

Table 6.5.1. Bioactivity of processed pro IL-1 beta molecule

intracellular precursor or IL-1 beta mRNA levels was seen.

Stimulated THP-1 cells were used as the source of IL-1 beta propeptide. Processing activity by the lysates themselves, added whole fresh cells or membrane preparations was detected. Inhibition of the appearance of the ~17 kDa form was demonstrated using aprotinin and the synthetic, 37 amino acid peptide that spans the cleavage site.

When recombinant pro IL-1 beta was used as reaction substrate, processing activity was found predominantly in membrane preparations. Prior stimulation of the cells did not alter their processing ability. The optimum time for the reaction was shown to be 1.5-2 hours. Synthetic peptides were unable to inhibit the production of ~17 kDa IL-1 beta in this system. Of a range of protease inhibitors only 3,4 dichloroisocoumarin was effective. The bioactivity produced during the processing reaction was determined in the EL4/CTLL assay, approximately 35% of the recombinant molecule was recovered as bioactive IL-1.

Cell lines were initially stimulated to establish a source of natural propeptide and of IL-1 beta processing activity. It was found that though each line was able to produce propeptide the mature molecule was only detectable in THP-1 cell supernatants following stimulation with PMA. From previous studies of IL-1 production by this line it has been shown that the secreted IL-1 beta form has a comparable N-terminal amino acid sequence to the native protein of untransformed cells (Matsushima *et al*, 1986b). Western analysis was performed on cells cultured for 24 hours. The U937 line has been described as only producing secreted IL-1 bioactivity at time points after 48 hours (Palacios *et al*, 1982).

The THP-1 line was therefore used for cellular IL-1 processing studies, and was stimulated with 100ng/ml PMA. The effects of characterised protease inhibitors on processing were determined. 1,10 phenanthroline at high concentrations reduced the level of IL-1 beta propeptide, this corresponded to a reduction in IL-1 beta message but not the mRNA of a cell cycle-independent gene. In culture supernatants aprotinin and leupeptin inhibited the appearance of the 17 kDa but not the 31 kDa IL-1 beta form. Inhibitory

activity was also seen with PMSF that reduced the extracellular levels of both IL-1 beta molecules. When protease inhibitors were tested for their effect on IL-1 beta message both PMSF and leupeptin treated cultures showed a reduction in the intensity of the IL-1 beta mRNA bands but not a cell cycle independent mRNA species, aprotinin did not affect IL-1 beta mRNA levels.

It is possible that the inhibitory effect on the production of 17 kDa IL-1 seen with PMSF and leupeptin is due to slower accumulation of the precursor resulting in reduced secretion, though lower cell-associated levels are not apparent by Western blotting. Aprotinin however appears specifically to reduce the extracellular levels of mature IL-1 beta, without affecting propeptide or mRNA levels. When the synthetic 37 amino acid peptide was tested in this system no inhibition of processing was seen (data not shown).

Aprotinin is a broad spectrum serine protease that acts to inhibit proteolytic activity at the cell surface or in the culture supernatant (Hewlett, 1990). As serum was not used in cultures it is likely that aprotinin inhibited a membrane or secreted protease derived from the cells themselves. Extracellular serine protease activity has previously been suggested to be involved in the processing of IL-1 beta propeptide (Auron *et al*, 1987). The presence of IL-1 beta precursor in the culture supernatant suggests that secretion precedes the processing event, but it cannot be absolutely established, with current techniques, that propeptide release is not a result of cell damage or death.

When the stimulated THP-1 cell lysate preparation was used as the source of IL-1 beta propeptide, freshly added cells gave the best processing. Low levels of processing were seen both by membrane preparations and the cell lysates. In all cases aprotinin inhibited the processing reaction as did the synthetic 37 amino acid peptide. This suggests that the processing enzyme may be secreted from live cells. Alternatively a membrane protease may be present that loses activity on disruption of the cells.

Using cell lysates as the source of propeptide IL-1 resulted in very low levels of ~17 kDa product that were difficult to detect even using a sensitive avidin-biotin system. We therefore continued the study using

recombinant pro IL-1 beta as the reaction substrate.

Using recombinant pro IL-1 beta little processing activity could be demonstrated in culture supernatants. Cell lysates showed some activity but the highest level of processing was found with membrane preparations. Using sonication to disrupt the cells it is possible that small liposome-like structures may form that would not sediment under the centrifugation conditions we used. Contamination of the lysate preparation with these liposomes may account for the low level of processing seen in this fraction.

Processing activity was seen in membrane preparations from two myeloid cell lines, THP-1 and HL-60. As it had been described that processing activity is co-inducible with the IL-1 beta propeptide (Black *et al*, 1989b), we compared the levels of ~17 kDa product in PMA stimulated and unstimulated THP-1 cells. Our enzyme preparation was not affected by cell stimulation, and therefore appears to be constitutively expressed at least in this cell line. The membrane processing activity was shown to be sensitive to heating consistant with the notion that processing is enzyme-mediated.

A number of immunoreactive IL-1 beta bands were seen following the processing reaction, one of which co-migrated with mature recombinant IL-1 beta. That the co-migrating band represents the mature IL-1 beta molecule cannot be established by Western blotting. Differences of a few amino acids at the N-terminus would not necessarily result in observed differences in the migration of the bands. To confirm the identity of the ~17 kDa form N-terminal sequencing would be required.

A time course reaction was performed to establish the optimum time for appearance of the ~17 kDa band. Recombinant material in tissue culture medium slowly degraded during the course of the incubation. The cell lysate preparation showed some processing activity, evident at around 3 hours. The processing reaction by membrane preparations showed the optimum time of incubation to be 1.5-2 hours, after this the product gradually degraded.

Inhibition of recombinant pro IL-1 beta processing by membranes was not seen using any of the synthetic peptides. A number of the protease inhibitors were seen to alter the intensity of some of the high molecular weight IL-1

beta intermediates, but only 3,4 dichloroisocoumarin prevented the production of the ~17 kDa band. 3,4 DC is a potent inhibitor of serine protease enzymes (Harper *et al*, 1985).

It has been suggested that elastase may account for much of the IL-1 beta processing in membrane preparations, we therefore used a specific inhibitor (elastatinal) to remove its activity. Elastatinal did not, however, alter the intensity of the ~17 kDa IL-1 beta band when included in the reaction.

The relationship of the different sized IL-1 beta bands seen is not clear. It is possible that some of the high molecular weight material may represent intermediates in the production of the ~17 kDa form, or that these are the end products of other enzyme activities present. Inclusion of protease inhibitors or synthetic peptides in the reaction resulted in altered banding patterns at high molecular weights, this may be due to the inhibition of non-specific proteolytic activity. Further studies in which groups of protease inhibitors are included may identify any intermediates in the specific processing reaction, and reduce the production of non-specific cleavage products. This may also prevent the spontaneous degradation of the ~17 kDa product.

Analysis of the bioactivity produced as a result of processing was determined by EL4/CTLL assay. The IL-1 beta propeptide showed no activity, but a considerable biological response was seen with its processed products. How much of the bioactivity was due to mature 17 kDa IL-1 beta could not be established, as processing at sites slightly upstream of the authentic N-terminus has been shown to result in recovery of some bioactivity (Black *et al*, 1988). The level of bioactivity in processed IL-1 is dependent on how near to the optimal N-terminus (between residues 116 and 117) the precursor is cleaved. It has been reported that cleavage between Val^{114}-His^{115} can also result in full biological activity in the EL-4 thymoma assay (Hazuda *et al*, 1989).

There are various reports concerning the identity of the processing enzymes that act on pro IL-1 beta. An early report isolated the precursor

molecule and showed that its degradation to lower molecular weight forms was effected by a serine protease that was sensitive to the action of PMSF (Auron *et al*, 1987). More recent studies have used recombinant pro IL-1 beta as the substrate for reactions. An initial report suggested that techniques used for isolation of the recombinant precursor may result in loss of the native conformation of the molecule (Black *et al*, 1988). This was shown by the sensitivity of precursor to complete degradation by membrane preparations within the incubation period. According to these criteria our pro IL-1 preparation would appear to be in its usual conformation as two hour incubation with processing preparations led to the production of distinct bands recognisable by Western blotting.

In contrast to our results Hazuda *et al* (1988) were not able to detect processing activity by whole monocytic cells, but from cell fractionation experiments localised activity to the cell lysate or peripheral membranes. Black *et al* (1988) have demonstrated IL-1 beta processing activity in isolated KG-1 membrane, that results in two distinct products one of which co-migrates with mature IL-1 beta. The activity was only found in cells of myeloid lineage. This group (Black *et al*, 1989b) have also reported a cytosolic protease in THP-1 cells that is co-induced with IL-1 beta. This activity could be inhibited by iodoacetate but not by other factors including PMSF and pepstatin, suggesting the presence of a cysteine protease activity. Kostura *et al* (1989) have also identified a cytosolic enzyme able to cleave pro IL-1 beta at the correct site, as determined by amino acid sequencing. The enzyme was not able to cleave a modified IL-1 beta precursor in which Asp^{116} was converted to an Ala residue.

Other processing activities have been shown to cleave the propeptide slightly upstream of the authentic site, it has however been suggested that these may have biological relevance particularly in an inflammatory environment (Hazuda *et al*, 1990). Collagenase, cathepsin G and elastase all generate a considerable amount of biological activity from pro IL-1 beta, and are known to be produced at sites of inflammation. Release of IL-1 beta precursor *in vivo* may lead to the rapid generation of a bioactive form as a

result of extracellular proteolytic activity. Black *et al* (1989a) have described a secreted processing activity in monocyte culture supernatants. The enzyme is a pre-aspartate specific protease, that leaves one additional amino acid at the N-terminus. Such a cleavage event would be expected to result in significant levels of bioactive IL-1 beta.

We have identified two processing activities, one is seen to cleave IL-1 beta in whole cell cultures and cell lysate preparations. This activity is only demonstrable when using a highly sensitive avidin-biotin detection system for IL-1 beta. The activity appears to be either secreted or associated with the cell surface, and is likely to be a serine protease that exhibits sensitivity to aprotinin. The second factor is a THP-1 membrane activity that is able to process the recombinant pro IL-1 beta molecule. The enzyme is also likely to be a serine protease but with different protease inhibitor sensitivity.

Only the secreted processing activity was sensitive to the inhibitory effect of a synthetic peptide that spans the processing site. It is possible that the peptides do not share the epitopes required for binding of the membrane associated enzyme activity. Small peptide molecules, however, are known to adopt a number of different conformations *in vitro*. It may be that sufficient levels of peptide in the correct conformation are not available to inhibit the processing enzyme by substrate competition. When using cell lysate as the source of pro IL-1 beta factors such as chaperon proteins may be able to retain the conformation of synthetic peptides in a suitable state to interact with the processing molecule. Small peptide molecules of 8-12 residues have been shown to bind to hsc70 and BiP, two members of the heat shock protein family (Flynn *et al*, 1989).

Other studies using synthetic peptides as substrates and inhibitors of proteolytic processing reactions have shown that alteration of an amino acid residue at the cleavage site results in a considerably better affinity for the enzyme involved. One group designed peptides in an attempt to inhibit the processing activity of the HIV-1 protease (Moore *et al*, 1989). Use of the modified peptide *in vitro* has been effectively shown to prevent processing of

structural proteins of the virion core and of a number of essential replicative enzymes (Meek *et al*, 1990). It is possible that modification of the synthetic IL-1 beta peptides may also increase their inhibitory effectiveness.

It has been suggested that IL-1 alpha and beta precursors are post-translationally modified *in vitro* by either phosphorylation or myristylation (Kobayashi *et al*, 1988; Bursten *et al*, 1988). Interleukin 1 alpha has been reported to show greater phosphorylation and this has been linked to either enhanced stability or protease susceptibility by two different groups (Kobayashi *et al*, 1988; Beuscher *et al*, 1988). Myristylation has been suggested to direct proteins to particular intracellular locations, this may bring IL-1 molecules into the proximity of particular processing enzymes or secretory pathways. The potential effects of post-translational modifications on IL-1 beta processing have as yet not been addressed.

We have described two pro IL-1 beta processing activities that are distinguishable by their sensitivities to common protease inhibitors. Both however appear to be serine proteases. The secreted enzyme form could also be inhibited by a synthetic 37 amino acid peptide designed around the pro IL-1 beta processing site. The other processing activity could be localised to isolated membrane preparations.

The membrane processing activity described may be involved in the normal IL-1 beta secretory process. It has be shown by many workers that mature IL-1 beta is not found intracellularly and it has, therefore, been suggested that IL-1 beta processing and secretion are closely linked (Black *et al*, 1988). A processing activity situated at the plasma membrane may be able to promote both cleavage and secretion, accounting for the lack of intracellular mature IL-1.

The activity that is found predominantly in culture supernatants may be involved in cleaving pro IL-1 beta that is released from the cell. It is not apparent whether IL-1 precursor molecules are normally released as part of the secretory process, or as a result of damage to the plasma membrane. The

extracellular enzyme activity may be involved in the processing of IL-1 beta propeptide at a localised inflammatory site, such as in RA synovial fluid, in a way similar to that described by Hazuda *et al* (1990). The enzymes secreted at an inflammatory locus may also have a broader role in the destructive processes seen in inflammatory diseases. By converting released, inactive IL-1 to a bioactive form further amplification of the inflammatory state may occur, causing further damage that may exceed the reparative capacity of the tissues involved. Specific inhibitory factors such as the synthetic peptide described here are, therefore, potentially important molecular models for the design of novel anti-inflammatory medicines.

7. CONCLUDING DISCUSSION

The development of reliable techniques for measurement of IL-1 in biological fluids has only recently become possible with the availability of immunoassays. The presence of IL-1 in the plasma of some normal individuals provides support for the suggestion that this cytokine is involved in normal physiological processes.

The source of circulating IL-1 in healthy individuals is unknown. The reported changes in IL-1 levels following exercise (Cannon and Kluger, 1983), and during the menstrual cycle suggest some regulated activation of a population of IL-1 producing cells. However it is possible that "normal" plasma IL-1 may be produced as a result of a sub-clinical inflammation or immune activation in a healthy individual.

Many reports have been published showing raised plasma or serum IL-1 levels, particularly of the beta form, in various disease states. The increase in circulating levels may result from systemic activation of the immune response or production at a localised site. In the case of RA a localised inflammatory reaction in the synovium may result in high concentrations of IL-1 in the joint. Some of this may then enter the circulation and trigger the systemic changes seen in rheumatoid disease.

The role of individual cytokines in the local inflammatory reaction is difficult to establish. It is becoming increasingly apparent that the effects of one particular cytokine *in vitro* are highly susceptible to the modulating effects of others. The *in vivo* environment is likely to be considerably more complicated, the network of interacting cytokines and other inflammatory mediators being difficult to analyse with current techniques.

In most cases the triggering event that leads to the production of sufficient IL-1 to result in localised destructive effects is unknown. It is possible that the damage seen in inflammatory disease is a result of a susceptibility in certain individuals to high IL-1 production based on cellular control mechanisms or inadequate negative feedback mechanisms.

Production of IL-1 in the course of normal physiological processes must be subject to control mechanisms that are able to regulate its potentially destructive effects. Feedback inhibition of IL-1 production has been

described in the case of the glucocorticoid system (Knudsen *et al*, 1987). Interleukin 1 has also been shown to prevent its own synthesis (Manson *et al*, 1988) and to be regulated by factors such as prostaglandins (Knudsen *et al*, 1986).

Factors able to modify IL-1 action following its production and secretion are also likely to play an important role in protecting biological systems from the unwanted actions of the cytokine. Our results show that plasma IL-1 is associated with factors that prevent detection of bioactivity and the recognition of certain immunoreactive epitopes. Assessment of the molecular weight of circulating IL-1 also indicates an interaction with other protein moieties.

We have described a circulating binding protein that is now known to prevent IL-1 beta binding to both the 80 kDa and 60 kDa IL-1 receptors, and is therefore a candidate molecule for modulating the cytokine's action *in vivo*. Little is known about the production the IL-1 binding protein, that the factor is released following stimulation of peripheral blood mononuclear cells (Symons *et al*, 1990) and its presence in plasma is indicative that it may have a role in the regulation of the systemic effects of IL-1 beta. The molecule is also found in inflammatory joint fluid suggesting a role in the control of the local inflammatory response.

Many soluble receptor molecules, both *in vitro* and *in vivo*, have been studied. Soluble receptors represent a readily available source of inhibitory activity. They may either be synthesised specifically as an alternative receptor form or cleaved from the surface in response to suitable stimuli. As yet little is known concerning the enzymes responsible for releasing membrane bound receptors in this manner, or the particular stimuli that activate the cleavage event. Surface receptors, however, may represent a rapidly mobilisable source of inhibitory activity.

A solubilised receptor is likely to represent a highly specific antagonist molecule to its ligand. That the soluble IL-1 beta binding protein retains comparable ligand affinity to the membrane receptor and inhibits the IL-1 beta/receptor interaction *in vitro*, suggests that it will

compete effectively for the cytokine in an *in vivo* situation. The presence of the binding protein in the plasma of healthy individuals indicates that it may take part in the normal regulatory processes controlling the actions of IL-1 beta.

The activities of IL-1 are likely to be regulated at multiple levels and another potential means of control is through the cloned inhibitor molecule. The role of the cloned IL-1 inhibitor (receptor antagonist) *in vivo* has been better characterised. The receptor antagonist has been shown to have inhibitory effects in various systems. However little is known concerning the regulation of its production. *In vitro*, the IL-1 inhibitor is secreted by aged monocytes (Roux-Lombard *et al*, 1989) indicating that the tissue macrophage may be a likely source *in vivo*. Monocytes activated by immune complexes also produce the factor (Arend *et al*, 1989b), indicating the possibility of a systemic cellular source following immune activation.

The involvement of non-specific factors such as alpha $_2$M need also to be considered. Though the interaction with IL-1 does not appear directly to inhibit biological activity, complexes may be more rapidly removed from the circulation. One report suggests that IL-1 preferentially binds to the "slow" form of alpha $_2$M (James *et al*, 1990). Activation to the "fast" form reveals a specific binding site recognised by macrophages and facilitates removal from the circulation. This suggests that the IL-1 interaction with alpha $_2$M does not promote clearance of this cytokine. There are reports of alpha $_2$M protecting cytokines from the destructive effects of proteolytic enzymes (Matsuda *et al*, 1989). Alpha $_2$M is a major serum protein and is produced predominantly by the liver. It is found in extravascular fluids such as lymph (James, 1990) and may therefore have a major role in modulating the systemic effects of IL-1. At a local level alpha $_2$M is produced by macrophages and so also has the potential to act as a regulatory molecule and control localised inflammatory processes at a site of tissue injury.

It has been demonstrated that radiolabelled IL-1 administered systemically is rapidly cleared from the circulation and localises in various tissues. The main organ of clearance is thought to be the kidney (Klapproth

et al, 1989). The role of factors such as alpha $_2$M and the IL-1 beta binding protein in tissue localisation and systemic clearance has not yet been determined.

Inhibition of the damaging effects of IL-1 by pharmacological agents could be effected by a number of mechanisms. An inhibitor could act by interacting with IL-1 itself to prevent association with the receptor, as appears to be the case with the IL-1 beta binding protein. The potential pharmacological properties of the engineered soluble 80 kDa receptor have been investigated (Fanslow *et al*, 1990), systemic administration of the sIL-1R was shown to prolong the survival of heart allografts in mice by neutralisation of the effects of endogenously produced IL-1. In a similar manner to the described physiological IL-1 inhibitor a factor could bind to the receptor molecule itself. The sequence of the IL-1 inhibitor shows 26% homology to IL-1 beta (comparable to the homolgy between the two IL-1 forms, Eisenberg *et al*, 1990). It is likely that the inhibitor bears a structural motif common also to the ligand molecules, that mediates interaction with the receptor. Another potential site for inhibition would be at the signal transduction level as has been reported for the synthetic peptide designed around the retroviral envelope protein p15E (Gottleib *et al*, 1989).

Efficient inhibition of IL-1 action could be effected by blocking its production, secretion or post-translational processing. As IL-1 beta is thought to be the predominant released form and is not active as the precursor molecule it is the more obvious target for pharmacological modulation. The cellular release of IL-1 is a poorly characterised event. As it appears possible that IL-1 precursors are released following cellular damage, blocking the action of a specific cellular processing event may not be sufficient to achieve a pharmacological effect. There is evidence that other proteolytic enzymes are able to cleave the IL-1 beta propeptide in a non-specific manner resulting in the recovery of different IL-1 molecules with some bioactivity. To be effective a potential inhibitor may need to block the processing of IL-1 beta by both a cellular enzyme and a variety of different extracellular proteolytic activities, often found a sites of

inflammation.

Though much is now known about the *in vitro* effects of IL-1 little is understood concerning the factors affecting its *in vivo* actions or the control of potentially damaging effects. The recent production of the recombinant receptor antagonist molecule will be a helpful tool in future studies as will the further characterisation of factors such as the soluble receptor molecules for IL-1 beta described here. What seems clear is that we are now only at the threshold of understanding the complex system of cytokines that co-ordinate the functions of the immune system.

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9. APPENDIX

9.1. CELL CULTURE

9.1.a. Mycoplasma testing

Cell lines were regularly tested for mycoplasma contamination using a Gen-probe detection system (Laboratory Impex). The test used a ^{3}H-labelled DNA probe specific for *Mycoplasma* and *Acholeplasma* ribosomal RNA. Briefly, 3 day culture supernatants were centrifuged at 200xg to remove cells, 2ml was then respun at 12000xg for 10 minutes and the media discarded. Two hundred microlitres of probe was added to the pellet and vortexed. At the same time positive and negative controls were prepared by adding 50ul of the solutions provided to 200ul of tritiated probe. During the hybridisation reaction tubes were floated in a water bath at 72° C overnight. The contents of each tube were then transferred to 7ml screw-cap scintillation vials (ICN Radiochemicals, High Wycombe) with 5ml of separation suspension, these were vortexed and incubated for a further 5 minutes at 72° C. Vials were centrifuged at 500xg, the supernatant discarded and the pellet washed twice: this involved adding 5ml of wash solution, resuspending the pellet and incubating at 72° C, the reaction was spun and decanted as previously described. The pellet was resuspended in 5ml of scintillant and each vial counted for 1 minute.

To determine total counts 5ml of separation solution was centrifuged and the pellet retained, as described for samples. Fifty microlitres of probe was added followed by scintillant and the vial was counted. Background counts were determined using 5ml of scintillation solution alone.

Any cell lines found to be contaminated with mycoplasma were treated using an antibiotic, BM-Cycline (Boehringer Mannheim). The antibiotics were supplied as two preparations; BM-Cycline 1 and BM-Cycline 2 which were dissolved in 10ml sterile PBS, this stock solution was stored at -20^{0}C. A three cycle treatment protocol was used, as recommended, in which 40ul of BM-Cycline 1 was added to each 10ml culture for three days and replaced by medium containing 40ul BM-Cycline 2 for a further four days. This was repeated three times with cultures being passaged as necessary at the start of each treatment. It was found that some lines were sensitive to the toxic

effects of the antibiotics, in these it was helpful to maintain cells at a slightly higher density than usual.

9.1.b. T Cell Growth factor production

The spleens were removed from young adult Wistar rats and the cells were dissociated into RPMI 1640 using two sterile frosted glass slides, The suspension was decanted, avoiding any large tissue clumps, and was centrifuged at 200xg for 10 minutes. Splenocytes were cultured at $1x10^7$ with 5ug/ml concanavalin A (Sigma) in serum free RPMI for 24 hours. The supernatant was harvested and the cells spun down. Conditioned media was recentrifuged at 1800xg. Aliquots of TCGF were stored at -20° C, and before use were filtered through a 0.22um filter.

9.2. CHEMICAL CROSS-LINKING SOLUTIONS

Binding buffer - 1x RPMI-1640, 1% bovine serum albumin, 0.1% sodium azide (Sigma), 20mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid) (Hepes, Sigma) at pH 7.4.

Quenching solution - 10mM Tris-HCl at pH 7.5, 1mM Na_2EDTA.

Lysis buffer - 1% SDS, with protease inhibitors; 10mM PMSF, 2mM EDTA, 2mM pepstatin, 2mM 1,10 PA.

9.3. PROTEIN PURIFICATION COLUMNS

9.3.a. Purification column buffers

Phosphate buffered saline - made up as 10x concentrated stock; 1.3M NaCl, 0.07M disodium phosphate (Na_2HPO_4, BDH) and 0.03M monosodium phosphate (NaH_2PO_4, BDH).

100mM sodium phosphate buffer - 100mM solutions of both Na_2HPO_4 and NaH_2PO_4 were prepared and the di-sodium preparation taken to the required pH using the mono-sodium solution.

IL-1 beta affinity column wash steps - 10 column volumes was used each of; 0.1M Tris-HCl pH 7.5/ 1.0M NaCl, 1.0M Tris-HCl/ 0.1M NaCl/ 10% ethylene glycol, and 0.1M Tris-HCl/ 0.1M NaCl.

9.3.b. Treatment of purification columns

Regeneration of wheat germ agglutinin column- The column was first washed with 10 volumes of 0.1M Tris-HCl/ 0.5M NaCl at pH 8.5, followed by 0.1M sodium acetate (BDH)/0.5M NaCl at pH 4.5 and was then re-equilibrated with PBS.

Hydration of Thiol Sepharose-4B - One gram of freeze dried gel was prepared by washing in 100mM sodium phosphate buffer (200ml/g of gel) at pH 8.0. The wash was done on a scintered glass filter at room temperature over 15 minutes.

9.4.a. MOLECULAR BIOLOGY TECHNIQUES

All techniques were done using sterile tissue culture plastics or glassware that had been either baked for 4 hours at 250° C, or treated by an overnight soak in diethyl pyrocarbonate (DEPC) water and then autoclaved. Experiments were done using gloves and care was taken to prevent RNAse contamination.

Water was prepared by adding 5ml of DEPC (Sigma) to each litre of distilled water and stirring overnight. To remove remaining DEPC, which may interfere with subsequent reactions, treated water was autoclaved.

9.4.b. Reagents

Oligolabelling buffer - Prepared by mixing solutions A, B, and C at a ratio of 100: 250: 150. Solution O, 1.25M Tris-HCl, 0.125M magnesium chloride (MgCl, BDH) at pH 8.0. Solution A, 1ml solution O, 18ul 2-mercaptoethanol, 5ul of each triphosphate solution (Boehringer Mannheim). Solution B, 2M Hepes (free acid) to pH 6.6 with 4M sodium hydroxide. Solution C, hexadeoxyribonucleotides (Pharmacia) in TE at 90 OD units/ml.

Tris-EDTA (TE) - 100mM Tris-HCl pH 7.4, 10mM EDTA.

Saline sodium citrate (SSC) - 20x solution; 0.3M sodium citrate (BDH), 3M NaCl.

Phosphate buffered saline sodium citrate (SSCP) - 20x solution; 0.3M sodium citrate, 2.4M NaCl, 0.4M sodium dihydrogen phosphate dihydrate to pH 7.4 with sodium hydroxide.

Denharts - 50x solution; 1% BSA, 1% Ficoll (Sigma), 1% polyvinyl pyrolidine (Sigma).

9.5. PROTEIN ANALYSIS SOLUTIONS

Loading buffer - 60mM Tris-HCl, 20% glycerol (Sigma), 4% SDS, 20% saturated bromophenol blue (Sigma).

Blocking buffer - 20mM Tris-HCl pH 7.4, 0.15M NaCl, 5% BSA, 0.2% Tween 20.

Second antibody buffer - PBS, 1% BSA, 1mM magnesium chloride (BDH), 0.1% Tween 20.

Substrate buffer - 0.1M Tris-HCl pH 8.8, 1mM magnesium chloride, 0.1M zinc chloride (Sigma).

Tris/Tween buffered saline (TTBS) - 100mM Tris-HCl pH 7.5, 0.15M NaCl, 0.1% Tween 20.

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CORRELATION OF PLASMA INTERLEUKIN 1 LEVELS WITH DISEASE ACTIVITY IN RHEUMATOID ARTHRITIS

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Summary The mean plasma level of interleukin 1 beta (IL-1 beta), measured by immunoassay, was significantly higher in 51 patients with rheumatoid arthritis (RA) than in 21 healthy controls of similar age. Further, in the RA group, plasma IL-1 beta correlated positively with Ritchie joint index, pain score, and erythrocyte sedimentation rate and correlated negatively with haemoglobin concentration. In individual patients with active disease who had serial measurements, plasma IL-1 beta also correlated with clinical disease activity. These results support the idea that IL-1 beta has a central role in the pathogenesis of RA.

Introduction

IL-1 molecules are polypeptides with many biological activities related to host defence and tissue remodelling,[1] and interest has arisen in the pathogenic potential of IL-1 in inflammatory and immune diseases.[2] Two forms of human IL-1 (alpha and beta) are encoded by separate genes with inducible expression in macrophages, lymphocytes, endothelial cells, and other cell types. The initial product of each is a propeptide of 31 kD from which mature peptide of 17 kD is processed.[3-5] IL-1 alpha and beta have only 26% aminoacid homology but compete equally for receptors on target cells[6] and have similar biological activities.[5] In resting blood monocytes IL-1 mRNA is undetectable but after cellular activation it accumulates rapidly and IL-1 beta is usually the predominant form.[3,5]

Many activities of IL-1 are relevant to rheumatoid arthritis (RA).[7] IL-1 increases the release from synovial cells of vasoactive agents and mediators of tissue damage (eg, prostaglandins, proteinolytic enzymes, and reactive oxygen molecules)[1,5] and is a powerful stimulus of bone[5,8] and cartilage[1,5,9] resorption. It also induces the acute-phase response[10] and fever[1,5,11] and may potentiate chronic inflammation by induction of lymphocyte growth factors such as interleukin 2 and its receptor.[12]

Much evidence[13] has implicated IL-1 in the pathogenesis of RA: high levels are found in synovial exudates;[14-16] RA synovial cells spontaneously produce biologically active IL-1 in vitro;[17] in-situ hybridisation of mRNA shows that IL-1 gene expression is activated in vivo in RA synovial cells;[16] and in laboratory animals a single intra-articular

injection of IL-1 produced synovial fluid leucocytosis and increased proteoglycan breakdown.[18]

Despite the weight of circumstantial evidence for IL-1 as a pathogenic mediator in RA, it has not previously been possible to correlate IL-1 levels in vivo with clinical disease activity in patients because IL-1 measurement in blood and other biological fluids has been complicated by interfering factors.[14,15] By means of a sensitive immunoassay combined with a single plasma extraction procedure we have now been able to measure circulating levels of immunoreactive IL-1 beta in patients with RA.

Methods

Patients with definite or classical rheumatoid arthritis[19] attending a rheumatology outpatient department or admitted to a rheumatology ward were graded for disease activity by Ritchie joint index,[20] duration of early morning stiffness, and visual analogue pain score. At the same time, blood was taken for haemoglobin concentration, white cell count, platelet count, erythrocyte sedimentation rate (ESR), and rheumatoid factor titre as part of the normal management. Blood samples for plasma preparation were taken into EDTA (5×10^{-3} mol/l) tubes (Labco) containing aprotinin (bovine lung 15–30 U/ml; Sigma) at 0·67 U/ml. Plasma was separated from cells and platelets immediately by centrifugation at 400 *g* and then at 10 000 *g* and stored in 500 µl samples at −20°C until tested for IL-1 beta content. All patients received non-steroidal anti-inflammatory drugs (NSAID), and some received second-line antirheumatic drugs, including intra-articular steroids.

IL-1 beta Measurement

Before IL-1 measurement, samples were treated according to the method of Cannon et al.[21] Plasma samples were thawed and extracted twice with chloroform (BDH, Paisley) by addition of 2 volumes of chloroform to 1 of plasma, mixing for 5 minutes, and then centrifugation at 10 000 *g* and removal of the aqueous phase for IL-1 beta testing in an enzyme-linked immunoabsorbent assay (IL-1 beta ELISA; Cistron Biotechnology/Laboratory Impex). In our laboratory,[16] this assay is sensitive to 20 pg/ml IL-1 beta, and has parallel dilution characteristics, less than 12% interassay variation, and detection recovery of added IL-1 beta in RA plasma of 82% after extraction (n = 10; mean 82·2 SEM 2·7%; difference from recovery in extracted normal plasma not significant). There is no cross-reactivity with IL-1 alpha. The ELISA test is commercially available. To 94-well microtitre plates coated with monoclonal antibody specific for IL-1 beta were added either

INTERLEUKIN-1 BETA IN EXTRACTED PLASMA

	Plasma IL-1 beta (pg/ml)	
—	Control	RA
n	21	51
Range	20–78	20–230
Median	50	90
Mean	44·7	98·2*
SEM	4·4	7·9

*Controls *vs* RA: $t = 4{\cdot}28$; $p = < 0{\cdot}0001$.

extracted plasma or IL-1 beta standards, with incubation at 37°C for 2 hours. After washing, a polyclonal rabbit anti-human IL-1 beta antiserum was added, with a further 2 hours of incubation. Horseradish-peroxidase-labelled goat anti-rabbit IgG was added, followed by enzyme substrate (*o*-phenylenediamine) to produce a chromogenic reaction. For colorimetry we used a scanning spectrophotometer (micro ELISA reader; Dynatech) at 490 nm. Concentrations of IL-1 beta were obtained from a standard curve derived from homogeneous human recombinant IL-1 beta.

Statistical Methods

Mean values were compared by Student's *t*-test and correlations were assessed with the Spearman rank correlation coefficient.

Results

IL-1 beta Levels in Plasma

In 51 patients with RA (age 26–63, mean 40 years) the mean IL-1 beta level in extracted plasma was significantly higher than in 21 healthy individuals (age 26–54, mean 38 years) (table). In 35 outpatients with RA and 11 healthy subjects, plasma IL-1 beta was measured both before and after the extraction procedure. In the RA patients, the unextracted mean was 54·5 pg/ml, increasing to 85·6 pg/ml after extraction ($p < 0{\cdot}001$), and in the controls it was 20·3 pg/ml (below detection, an arbitrary value of 19 pg/ml was assigned) increasing to 33·4 pg/ml after extraction ($p < 0{\cdot}05$). There was no obvious relation between plasma IL-1 beta level and therapy with any particular agent but the variety of antirheumatic drugs used in these patients precluded confident analysis.

Having seen that the extraction procedure increased the detection of plasma IL-1 beta both in RA patients and in controls and that plasma IL-1 levels were higher in patients, we then sought correlations between plasma IL-1 beta

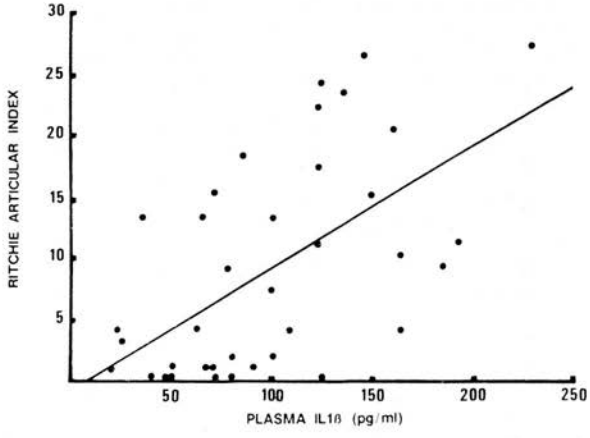

Fig 1—Correlation between plasma IL-1 beta concentration and Ritchie articular index in patients with RA.

$n = 37$; $r = 0{\cdot}572$; $p < 0{\cdot}001$.

concentration and conventional indices of disease activity in the RA patients.

Plasma IL-1 beta Correlations with Disease Activity

In samples from different RA patients, IL-1 beta concentration in extracted plasma correlated significantly with: Ritchie joint score (fig 1; $r = +0{\cdot}572$, $p < 0{\cdot}001$); duration of morning stiffness ($r = +0{\cdot}473$, $p < 0{\cdot}005$); pain

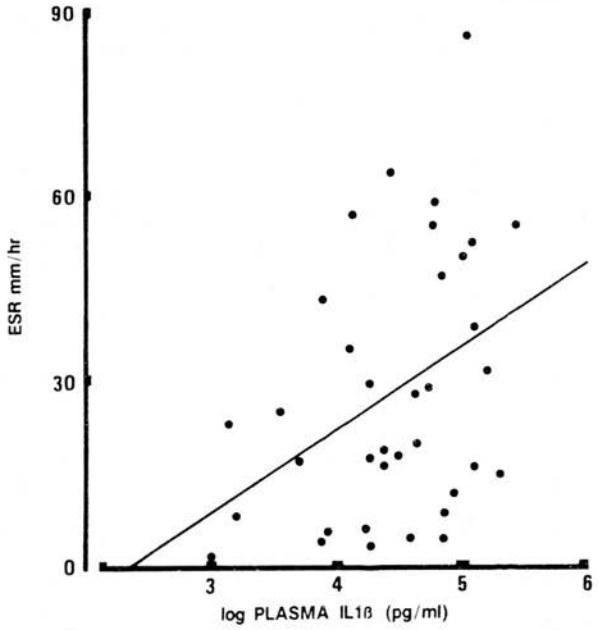

Fig 2—Correlation between plasma IL-1 beta (natural log scale) and ESR in patients with RA.

n = 35; $r = 0{\cdot}358$; $p < 0{\cdot}05$.

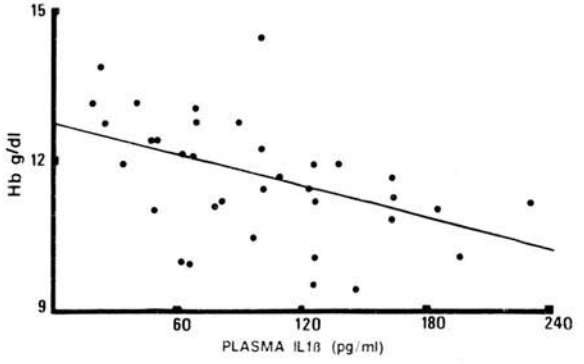

Fig 3—Negative correlation between plasma IL-1 beta and haemoglobin concentration in patients with RA.

n = 36; $r = -0{\cdot}53$; $p < 0{\cdot}001$.

score ($r = +0{\cdot}605$; $p < 0{\cdot}001$); ESR (fig 2; $r = +0{\cdot}358$, $p < 0{\cdot}05$); and haemoglobin (fig 3; $r = -0{\cdot}53$, $p < 0{\cdot}001$). There was no significant cross-sectional correlation with platelet count, rheumatoid factor titre, or white cell count.

Serial Measurements of IL-1 beta in Individual Patients

There was a large variation in the plasma IL-1 beta level between individual RA patients but, in hospital patients

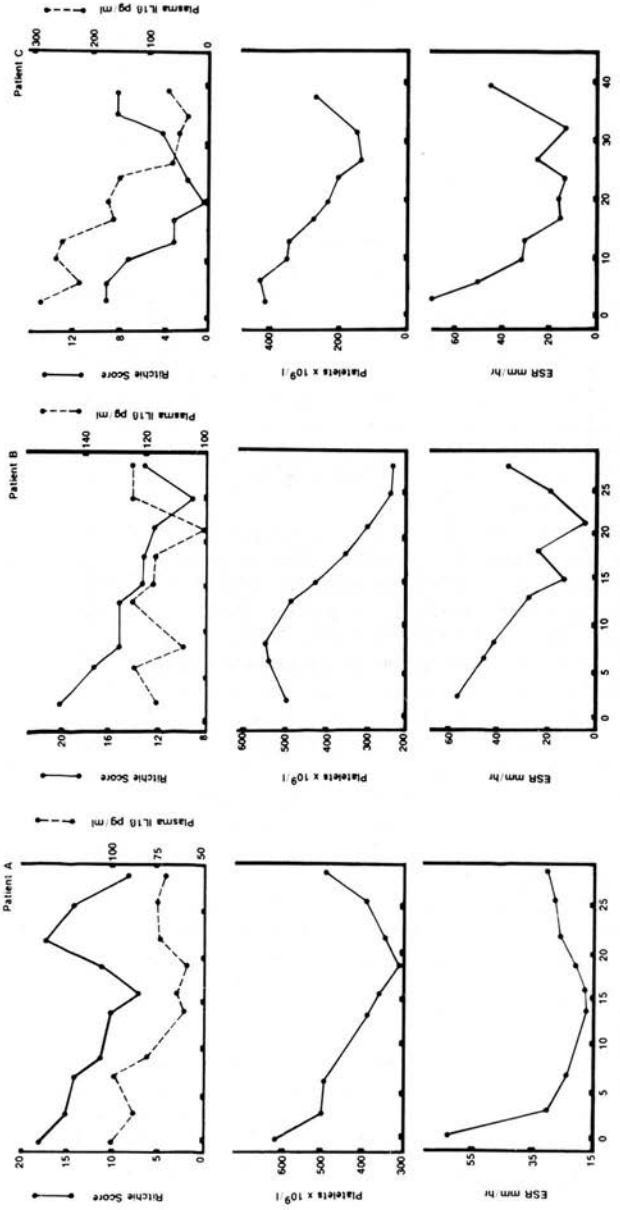

Fig 4—Serial measurements of plasma IL-1 beta, Ritchie articular index, platelets, and ESR during hospital admissions in 3 patients with active RA who underwent secondary flares in disease activity after initial improvement.

Top panel: plasma IL-1 beta (broken line), Ritchie index (solid line). Middle panel: blood platelet count. Lower panel: ESR.

tested at different times over several weeks, IL-1 beta levels were related to clinical disease activity. For example, fig 4A shows serial plasma IL-1 beta, Ritchie index, platelet count, and ESR in a patient admitted with acute exacerbation of RA. After initial improvement there was an increase in joint pain, stiffness, and swelling as reflected in the Ritchie index. In this individual, concurrent plasma IL-1 beta levels correlated significantly with ESR ($p = 0{\cdot}02$) and platelets ($p = 0{\cdot}02$). The correlations with Ritchie index ($p = 0{\cdot}08$) and pain score ($p = 0{\cdot}07$) were not statistically significant. Fig 4 B and C shows similar results when the same indices were measured serially in two further patients. There were no statistically significant correlations in patient B but, in patient C, plasma IL-1 level correlated with: platelets ($p < 0{\cdot}005$); white cell count ($p < 0{\cdot}005$); pain score ($p < 0{\cdot}01$); and ESR ($p < 0{\cdot}05$).

Discussion

We have shown that, in patients with RA, immunoreactive levels of IL-1 beta in extracted plasma are higher than in healthy volunteers. More importantly, the concentration correlated with clinical (Ritchie index, morning stiffness, pain score) and laboratory (ESR, haemoglobin) evidence of disease activity within the group of patients. In individuals tested at different times, correlations were found with pain score, white cell count, platelet concentration, and ESR and similar trends were seen in the Ritchie index.

Immunoreactive IL-1 beta is not necessarily biologically active since forms of the molecule (both larger and smaller than the mature peptide) may fail to bind the IL-1 receptor but retain sites for antibody recognition. Further, it seems that inhibitory molecule(s) may mask the immunoreactivity and bioactivity of IL-1 in unextracted samples.[21,22] Recently, an inhibitor of IL-1 bioactivity was reported in serum and urine of children with juvenile chronic arthritis.[23] However, the clinical correlations in our study suggest that plasma levels of immunoreactive IL-1 reflect production of biologically active IL-1 at sites of inflammation.

Transcription of the IL-1 gene seems not to be constitutive but occurs with cellular activation in most mesenchymal cell-types tested.[3,5] We have found mRNA for IL-1 beta, IL-1 alpha, and tumour necrosis factor by in-situ hybridisation in synovial sections from RA joints,[16] and others have identified IL-1 mRNA in synovial extracts.[24] These observations indicate that IL-1 gene expression is activated in vivo within the rheumatoid lesion.

The raised concentrations of IL-1 in RA are of particular interest in view of the biological responses that IL-1 stimulates. These include: chemotaxis and activation of inflammatory cells;[25] release of prostaglandins and collagenase from synovial cells;[26] stimulation of synovial cell mitogenesis[17] and hepatocyte acute-phase protein

synthesis;[27] resorption of bone and cartilage;[8,9] and potent effects on the immune system through induction of lymphokines and their receptors.[12,28] While some of these effects may represent a direct action on target cells, others are probably mediated by different cytokines (eg, interleukin 6) stimulated by IL-1.[29] These biological actions of IL-1 seem relevant not only to the chronic proliferative synovitis and cartilage/bone erosion of RA but also to the systemic acute-phase response and increased symptoms associated with exacerbation of disease.

Of interest also is the negative correlation reported here between plasma IL-1 beta and haemoglobin concentration. In RA, as in other chronic diseases, a refractory anaemia is common. For some time IL-1 has been known to have effects on iron metabolism that could contribute to anaemia,[1] but other effects of IL-1 might lead to inhibition of erythropoiesis. For example, IL-1 has been reported to suppress in-vitro colony formation of erythroid progenitor cells.[30]

The present study does not address the extent to which IL-1 beta accumulation is itself a reaction to increased inflammation, but sustained or high IL-1 release could be pathogenic irrespective of when it occurs in the chain of events that results in clinical disease.

Though the kinetics of distribution are not fully known, we have found (Eastgate J, Symons JA, Wood NC, Duff GW, unpublished) that the blood level of the related cytokine IL-1 alpha was not comparable with that of IL-1 beta in terms of correlations with disease indices in RA

The interactions with synergistic and antagonistic factors are likely to be complex, but taken together with its arthritogenic, catabolic, and immunoregulatory activities, the clinical correlations reported here support the idea that IL-1 is an important pathogenic mediator in RA and give impetus to the task of developing agents to manipulate its production or biological activities in vivo.

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Identification of an interleukin-1 beta binding protein in human plasma

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A covalent cross-linking technique was used to bind iodinated interleukin-1 (IL1) alpha and beta to plasma proteins. One specific IL1 beta binding protein was observed, that when cross-linked to ^{125}I-IL1 beta migrated to approximately 60 kDa on SDS-PAGE. The protein did not bind IL1 alpha. The 43 -kDa protein was partially purified using a wheat germ agglutinin affinity column. The isolated factor again specifically bound IL1 beta, and appeared to consist of single chain glycoprotein. The protein was heat stable and had a rapid association time with IL1 beta. This protein may be an important carrier molecule for IL1 beta in vivo.

Interleukin 1 alpha; Interleukin 1 beta; Soluble binding protein; Interleukin 1 receptor

1. INTRODUCTION

Interleukin 1 (IL1) is a polypeptide produced by activated leukocytes and other cells. It mediates a wide range of biological activities relevant to host defence. It increases production of T and B lymphocyte growth factors and stimulates other cells involved in inflammatory and immune responses. Additionally, it plays a role in connective tissue remodelling, haematopoiesis and is an important factor in the induction of fever and acute phase protein synthesis [1].

Two distinct forms of IL1 (IL1 alpha and IL1 beta) have been described [2]. Although the two peptides have only 26% homology, they compete for the same cellular receptor with equal affinity [3,4]. The murine IL1 receptor (IL1R) has recently been sequenced and cloned [5] and is a member of the immunoglobulin gene superfamily. The 319-amino-acid extracellular domain consists of two beta-pleated sheets connected by disulphide bonds and seven potential N-linked glycosylation sites, which account for the described size heterogeneity of the IL1R.

High expression of IL1 peptides appears to be a common phenomenon in many inflammatory diseases leading to extensive tissue damage [6]. Therefore, regulatory pathways must exist to limit IL1-mediated responses. IL1-stimulated prostaglandin E_2 production post-transcriptionally blocks monocyte expression of IL1 activity [7]. In addition, a large number of IL1 inhibitory molecules have been described (reviewed by Larrick [8]), although the mechanism of inhibition in most cases is largely unknown. The most highly characterised IL1 inhibitor described is produced by macrophages in response to GM-CSF [9] and immune complexes [10] and has a molecular mass of approximately 26 kDa. This inhibitor blocks both IL1 alpha and IL1 beta but not TNF alpha binding to cell-surface receptors. Other proteins may control the distribution and bioavailability of IL1 in vivo by acting as carrier molecules. For example, treatment of plasma with trypsin or methylamine allows complex formation between alpha 2 macroglobulin and IL1 beta through disulphide bonding [11].

In this paper we describe the presence of a novel IL1 binding protein in normal human plasma. Unlike the previously known IL1 inhibitors and IL1 receptor protein, the IL1-binding protein described here appears to have affinity specifically for IL1 beta and does not bind IL1 alpha.

2. MATERIALS AND METHODS

2.1. *Reagents*

Radioiodinated hr IL1 alpha and hr IL1 beta proteins were obtained from Du Pont (NEN Products; Hertfordshire, England). IL1 alpha was radioiodinated using the chloramine-T procedure to a specific activity of 90.4 μCi/μg (3.35 mBq/μg). IL1 beta was labelled with Bolton Hunter reagent to a specific activity of 158 μCi/μg (5.85 mBq/μg). Cold human recombinant IL1 peptides were a gift from Biogen S.A., human recombinant TNF alpha was a gift from Dr Guenther Adolf (Ernst Boehringer Institut fur Arzneimittel Forschung, A-1121 Wien, Austria). Covalent cross-linking reagents disuccinimidyl suberate (DSS), disuccinimidyl tartarate (DST) and ethylene glycolbis (succinamidyl succinate) (EGS) were obtained from Pierce and Warriner, Chester, England.

2.2. *Cell culture*

Murine 3T3 fibroblasts were cultured in RPMI-1640 containing

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penicillin (100 U/ml), streptomycin (100 μg/ml), glutamine (2 mM) and 5% foetal calf serum (FCS; heat inactivated; 56°C, 30 min). All cell culture reagents were obtained from Northumbria Biologicals (Northumbria, England).

2.3. *Covalent cross-linking and PAGE analysis*

Plasma or semi-purified protein (50 μl) was incubated overnight at 4°C with 10 ng/ml of ^{125}I-IL1 alpha or IL1 beta, with or without 100-fold excess of cold IL1 alpha, IL1 beta or TNF alpha. Subsequently, covalent cross-linker, DSS, DST or EGS (final concentration 1 mg/ml) freshly prepared in DMSO, was added and incubated at 4°C for 30 min. Cross-linked complexes were identified in 10% polyacrylamide gels according to Laemmli [12]. Samples to be tested under reducing conditions were treated with dithiothreitol (100 mM; DTT) and boiled for 10 min.

Monolayers of 3T3 fibroblasts in 25 cm^3 flasks were incubated for 4 h at 8°C with 1 ng/ml labelled IL1 alpha or IL1 beta in RPMI 1640 containing 1% BSA, 0.1% sodium azide and 20 mM Hepes, pH 7.4 (binding buffer) in a final volume of 5 ml. Specificity of binding was controlled by the addition of excess cold cytokine as above. Unbound radioactivity was removed by washing three times in protein-free, ice-cold binding buffer. Five mililitres of this buffer was then added to each flask, cross-linker (DSS) was added to a final concentration of 1 mg/ml and incubated on ice for 45 min. The cross-linker was then removed and replaced with 5 ml of quenching solution (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). The cells were scraped using a rubber policeman into the buffer and centrifuged at 800 × *g* for 10 min at 4°C. The cells were subsequently dissolved in 100 μl of 1% (w/v) SDS containing protease inhibitors PMSF (10 mM), EDTA (2 mM), pepstatin (2 mM) and 1,10 phenanthroline (2 mM). Samples were analysed by SDS-PAGE as described above. Iodinated cytokines were routinely assessed for self-association in cross-linking experiments.

2.4. *Partial purification and characterisation*

IL1 binding proteins were partially purified using a wheat-germ agglutinin sepharose 6MB column (Pharmacia LKB Biotechnology, England). Briefly, plasma was equilibrated by extensive dialysis against PBS and then loaded on the column using a flow rate of 4 ml/h. The column was subsequently washed with PBS/0.3 M NaCl, PBS/10% (v/v) ethylene glycol and finally PBS. Specifically bound glycoproteins were then eluted from the column with PBS containing 500 mM *N*-acetyl-glucosamine and 0.3 M NaCl. Two-millilitre fractions were collected and screened for IL1 bindig activity by covalent cross-linking.

Heat stability of the partially purified binding protein was tested by incubating aliquots for 30 min at temperatures ranging from 4°C to 70°C. Treated samples were then incubated with iodinated IL1 beta and cross-linked as previously described. Kinetics of binding of IL1 beta to the protein was tested by incubating labelled cytokine with binding protein for various lengths of time at 4°C, room temperature or 37°C before cross-linking.

3. RESULTS

3.1. *Covalent cross-linking*

Binding proteins were identified by cross-linking plasma previously incubated with iodinated IL1. When IL1 alpha cross-linked to human plasma was analysed by SDS-PAGE, no specific binding was observable (fig.1A). A small amount of material that just entered the gel showed binding, but this appeared to be non-specific. To demonstrate that ^{125}I-IL1 alpha retained receptor binding activity, we cross-linked it to 3T3 fibroblasts. Fig.2 shows that IL1 alpha specifically bound to its 80 KDa receptor on 3T3 cells.

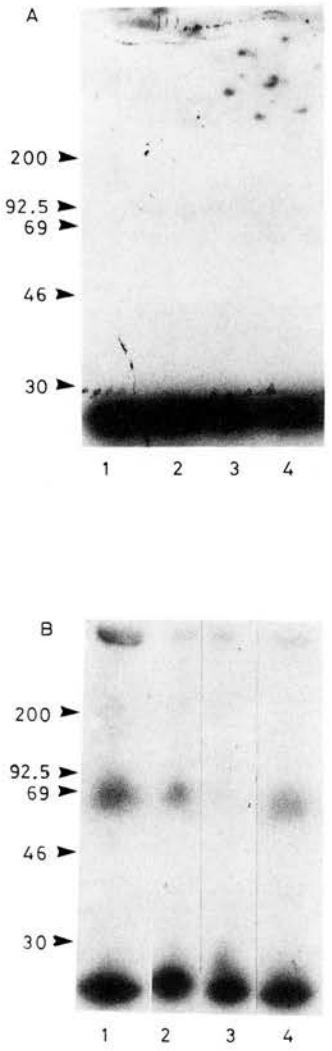

Fig.1. ^{125}I-IL1 alpha (A) and ^{125}I-IL1 beta (B) cross-linked to plasma (lane 1) with excess cold IL1 alpha (lane 2), IL1 beta (lane 3) and TNF alpha (lane 4).

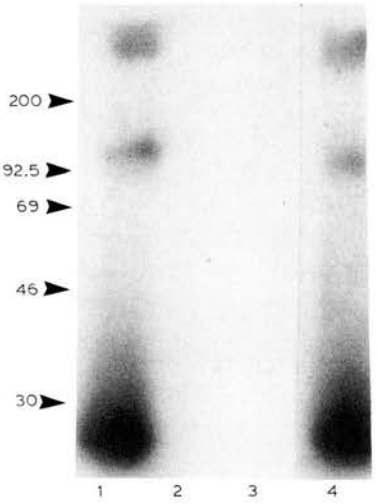

Fig.2. Cross-linking of iodinated IL1 alpha to 3T3 cells (lane 1) with excess cold IL1 alpha (lane 2), IL1 beta (lane 3) and TNF alpha (lane 4).

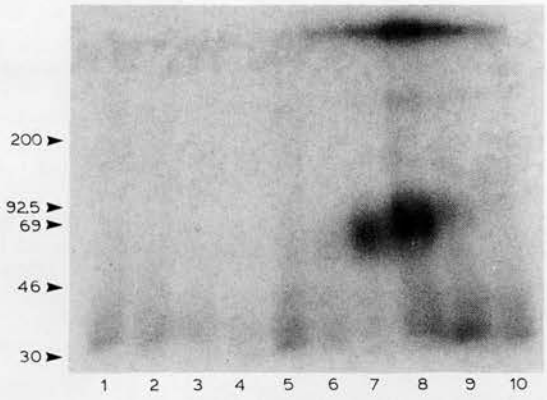

Fig.3. Cross-linking of ^{125}I-IL1 beta to fractions eluted from a WGA column.

In contrast when ^{125}I-IL1 beta was cross-linked to plasma, an IL1 beta/protein complex was observed to migrate at approximately 60 kDa (fig.1B). This binding was displacable by 100-fold excess of cold IL1 beta but not IL1 alpha or TNF alpha, and was seen with all three cross-linkers used. Some non-specific binding was also seen to high-molecular mass material.

3.2. *Purification and biochemical characterisation*

The 60 kDa IL1 beta/binding protein complex migrated as a broad band from 50–70 kDa suggesting the possibility of variable glycosylation. We therefore used lectin affinity chromatography for partial purification of the binding protein. Experiments revealed that the binding protein could be specifically eluted from a wheat germ agglutinin (WGA) affinity column. The result of this purification is shown in fig.3. The binding protein eluted from the column with *N*-acetyl glucosamine and when cross-linked to ^{125}I-IL1 beta, migrated at the same molecular mass as the unpurified plasma protein. The specificity of this protein for IL1 beta was retained (fig.4) as it failed to bind ^{125}I-IL1 alpha. Further ^{125}I-IL1 beta binding was only competed by 100-fold excess cold IL1 beta. Separation of plasma on the WGA column resulted in a 25-fold purification.

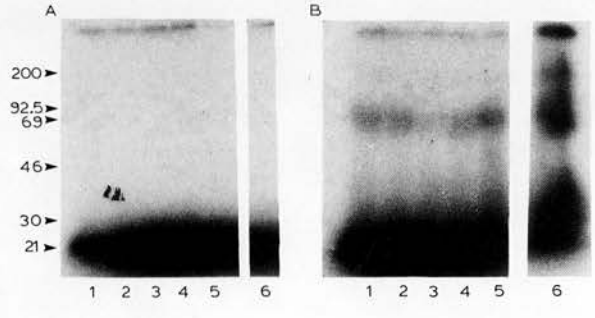

Fig.4. ^{125}I-IL1 alpha (A) and ^{125}I-IL1 beta (B) cross-linked to fraction 8 of the affinity column (lane 1), with excess cold IL1 alpha (lane 2), IL1 beta (lane 3) and TNF alpha (lane 4) and also under reducing conditions (lane 5) iodinated proteins are also cross-linked to plasma (lane 6).

Reducing SDS-PAGE resulted in the ^{125}I-IL1 beta/binding protein complex migrating at the same molecular mass as in non-reducing conditions (fig.4). Heat treatment of semi-purified binding protein before cross-linking showed that the ^{125}I-IL1 beta binding ability was retained even at 70°C (data not shown). Incubation of labelled IL1 beta with binding protein for various lenghts of time at 4°C, room temperature and 37°C indicated that rapid association occurred, with maximal binding after 30 min at 37°C (data not shown).

4. DISCUSSION

SDS-PAGE analysis of plasma cross-linked with iodinated IL1s shows an IL1 beta/binding protein complex of molecular mass 60 kDa suggesting the presence of a 43 kDa factor in human plasma capable of specific association with IL1 beta and not IL1 alpha. Partial purification using a WGA column isolated a protein that bound IL1 beta and was not affected by reducing conditions, suggesting the presence of a single polypeptide chain. The binding factor is thought to be a heat-stable glycoprotein with rapid association kinetics with IL1 beta. A high-molecular-mass factor is also seen to bind both labelled IL1 alpha and IL1 beta; this appears to be non-specific.

Vairous factors have been described as IL1 inhibitors [8], most of which are assayed by their ability to interfere with the co-mitogenic activity of IL1 on thymocytes. A well-characterised 23 kDa inhibitor affects both IL1 alpha and IL1 beta stimulation of fibroblast prostaglandin production and blocks iodinated IL1 alpha binding to its receptor [9]. Uromodulin, an immunosuppressive glycoprotein, inhibits IL1-induced T cell proliferation. This activity depends on intact glycosylation and it appears that IL1 itself binds to uromodulin through lectin-like binding [13]. It is unlikely that either of these inhibitors are comparable to the binding protein described here, due to molecular mass and specificity differences. A granulocyte-derived 45–70 kDa molecule [14] and a 40 kDa immunosuppressive protein from epidermal cells have been described to inhibit IL1-induced thymocyte co-mitogenesis and fibroblast proliferation [15]. However, none of these factors have been shown to be specific for either IL1 alpha or IL1 beta alone.

Some groups have suggested that the proteinase inhibitor alpha 2 macroglobulin (alpha 2M) may, in an activated form, bind IL1, accounting for its various immunosuppressive actions in vitro [11]. Activated alpha 2M has been found at high levels in synovial fluid of rheumatoid arthritis patients and also acts as a binding protein for IL6 [16]. Alpha 2M may account for the high-molecular-mass factor seen to bind non-specifically both IL1 alpha and IL1 beta in the present study.

The 43 kDa specific IL1 beta binding protein described here may represent a solubilised form of the IL1 receptor, but a recombinant form of the murine IL1R comprising only the extracellular domain retains equal affinity for IL1 alpha and beta [17]. Radiolabelled IL1 beta has a half-life in vivo of approximately 5–10 min following i.v. injection [18]. Despite the presence of IL1R on many cells circulating in the blood, IL1 beta is almost entirely associated with the plasma. Distribution studies indicate that apart from the liver and kidneys, IL1 beta distributes into all tissues to approximately the same extent. The specific binding protein described here may therefore act as a carrier molecule for IL1 in body fluids and may be an important determinant of tissue distribution and bioavailability in vivo. It may also explain in part the difficulty of measuring IL1 in body fluids. With the increasing interest in the potential of recombinant IL1 beta as a therapeutic molecule or as a target for pharmacological intervention, it becomes important to understand the factors that control its handling in vivo.

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A SOLUBLE BINDING PROTEIN SPECIFIC FOR INTERLEUKIN 1β IS PRODUCED BY ACTIVATED MONONUCLEAR CELLS

Julian A. Symons,* Julie A. Eastgate, Gordon W. Duff

Soluble interleukin 1 (IL 1) binding proteins were identified by gel filtration and covalent cross-linking of ^{125}I IL 1 in normal human serum and inflammatory exudate. High molecular weight ^{125}I IL 1 protein complexes occurred with both IL 1α and IL 1β, however, high molecular weight binding appeared to be non-specific. One specific IL 1β binding protein was observed to elute at approximately 100 kDa on gel filtration when bound to ^{125}I IL 1β. This complex migrated as a broad band at 60 kDa when covalently cross-linked and analyzed by SDS-PAGE. The protein did not bind ^{125}I IL 1α and ^{125}I IL 1β binding was only displaceable by excess cold IL-1β. The production of the specific IL 1β binding protein was assessed in a number of cell populations. Unstimulated peripheral blood mononuclear cells (PBMNC) did not produce the binding protein, but stimulation with phytohemagglutinin (PHA) caused production within 24 hr and binding protein levels remained elevated for up to 7 days. Stimulation with lipopolysaccharide (LPS) and IL 1α did not consistently induce synthesis of the binding protein. Ligand-binding studies were performed to compare solubilized EL 4 NOB.1 cell membrane IL 1 receptor (sIL 1R) with semi-purified IL 1β binding protein from pooled synovial fluid. The sIL 1R preparation bound ligand with an affinity of 168 pM while the IL 1β binding protein bound ^{125}I IL 1β with an affinity of 370 pM. This protein may function as an important carrier molecule for IL 1β and determine its distribution and kinetics in vivo.

Interleukin 1 (IL 1)α and IL 1β are polypeptides involved in the regulation of immune and inflammatory responses.[1] Although the two peptides have only 26% homology,[2] they compete with equal affinity for the same major class of cellular receptor.[3,4] Recently, the murine[5] and the human[6] IL 1 receptor (IL 1R) molecules have been sequenced and cloned. They share a 69% amino acid homology and are members of the immunoglobulin gene superfamily. In the murine IL 1R, the 319 amino acid extracellular domain consists of two β-pleated sheets connected by disulphide bands and seven potential N-linked glycosylation sites, which may account for the described size heterogeneity of the IL 1R.

IL 1 mediates a wide range of biological activities that, if not closely regulated, may lead to extensive tissue damage. High levels of circulating IL 1 are often seen by immunoassay in inflammatory diseases and correlate with other markers of disease activity.[7] In the absence of disease, homeostatic mechanisms must operate to limit IL 1 mediated responses. Many IL 1 inhibitory molecules have been described[8] but few have been characterized extensively. Recently, an IL 1 receptor antagonist has been sequenced[9] and cloned.[10] This protein is 26% homologous with IL 1β and 19% homologous with IL 1α, and binds IL 1R, but lacks IL 1 biological activity. The receptor antagonist is produced from monocytes, in response to immune complexes, IgG,[11] and granulocyte macrophage colony stimulating factor (GM-CSF)[12] and appears identical to an IL 1 inhibitor, which was isolated from the urine of febrile and myelomonocytic leukemia patients.[13] Other regulatory pathways of IL 1 include post-transcriptional control by prostaglandin E_2[14] and carrier molecules that bind to IL 1. α_2 macroglobulin (α_2 M) forms complexes with cytokines, including IL 1, following treatment with trypsin or methylamine to expose free thiol groups.[15] However, other plasma proteins with free thiol groups (such as fibronectin) do not bind IL 1, so other structural properties must be important.

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KEY WORDS: Interleukin, 1α/Interleukin, 1β/Soluble, binding protein/Interleukin, 1 receptor/Covalent, cross-linking.

In this paper, we describe a novel IL 1 binding protein, which is present in synovial inflammatory exudate and normal serum and that is produced by activated mononuclear cells. Unlike previously described inhibitors and binding proteins, this protein appears to have affinity specifically for the β form of IL 1 and does not bind IL 1α.

RESULTS

Identification of IL 1 Binding Proteins by Gel Filtration

In order to identify IL 1 binding proteins, synovial inflammatory exudate (SF) was incubated with ^{125}I IL 1 peptides and then separated by Sephacryl S-200 gel filtration (Fig. 1). When SF was incubated with ^{125}I IL 1β , two identifiable complexes were formed: a small peak of binding eluting at high molecular weight in the void volume and a larger peak of binding at 100 kDa, which accounted for approximately 16% of the available ^{125}I IL 1β. When the experiment was repeated with 500-fold excess cold IL 1β the binding activity eluting in the void was unchanged, however, the 100 kDa peak was reduced significantly. Incubation of SF with ^{125}I IL 1α also resulted in the formation of the high molecular weight complex, but no other distinct peaks were seen.

Identification of IL 1 Binding Proteins by Soluble Covalent Cross-Linking

A soluble covalent cross-linking procedure was used to characterize the binding proteins. After incubation of ^{125}I IL 1 peptides with the sample, complexes were covalently cross-linked with disuccinimidyl suberate (DSS) and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fig. 2A shows that when SF was incubated with ^{125}I IL 1α no specific binding proteins were observed. ^{125}I IL 1α-associated material that occassionally entered the gel (>500 kDa), was observed, however, this was not affected by excess cold IL 1 peptides. In contrast, when ^{125}I IL 1β was covalently crosslinked to SF proteins, several molecular weight species were seen (Fig. 2B). As with ^{125}I IL 1α a high molecular weight band (>500 kDa) was seen but this was not displaceable with either excess IL 1α, IL 1β or TNF α. While the major ^{125}I IL 1β protein complex migrated as a broad band at approximately 60 kDa, ^{125}I IL 1β binding to this complex was not displaceable by excess cold IL 1α or TNF α, but was completely displaceable by excess cold IL 1β. A fainter band was seen at 150 kDa, which was also displaceable with only excess cold IL 1β, however, this band was not seen in reducing conditions, where the 60 kDa band was unchanged (results not shown). Binding of ^{125}I IL 1β in

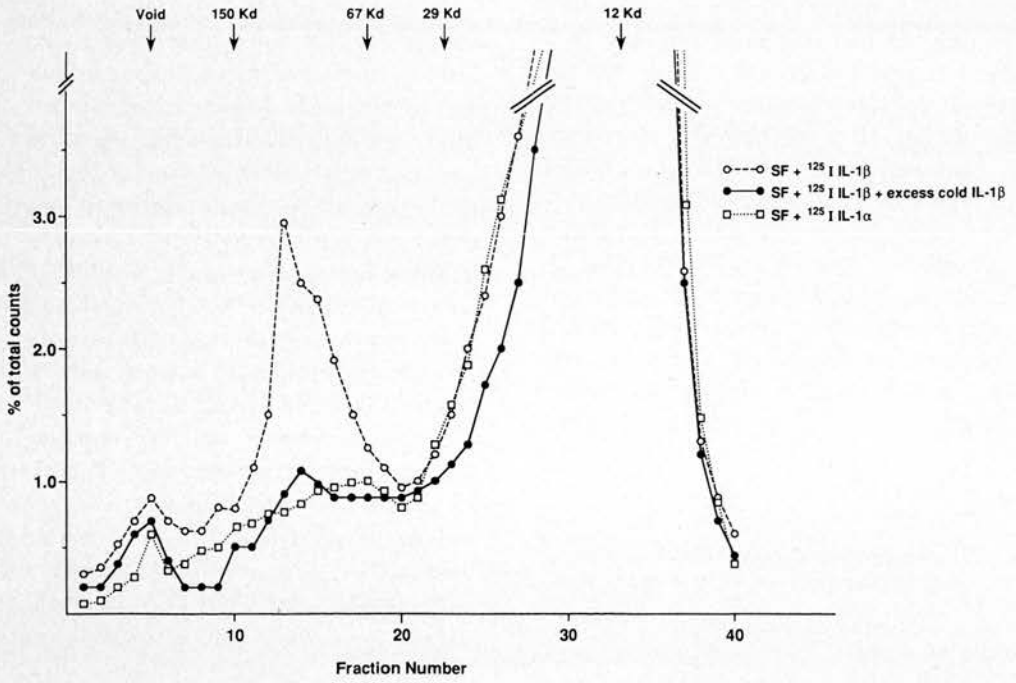

Figure 1. Gel filtration of IL 1 binding proteins.

Synovial fluid was incubated with either ^{125}I IL 1β or ^{125}I IL 1α and then subjected to gel filtration through Sephacryl S-200. Results are expressed as percentage of total counts in each fraction. Total counts loaded in each case were for ^{125}I IL 1β 65,667 (O—O), for ^{125}I IL 1β plus 500 x excess cold IL 1β 68,228 (●—●) and for IL 1α 161,844 (□—□) cpm. Results are representative of three different synovial fluid samples.

the 60 kDa complex, but was not displaceable by a variety of other cytokines, which include IL 2, GM-CSF, IL 4, IL 6, TNF β, or γ IFN. The binding protein complex, seen at 100 kDa on gel filtration of SF (Fig. 1), was covalently crosslinked and analyzed by SDS-PAGE, where it migrated at 60 kDa (data not shown).

Analysis of ^{125}I IL 1β binding to serum proteins by covalent crosslinking was also performed (Fig. 3). A similar pattern of binding was seen to that observed in SF.

To confirm that the iodinated IL 1α, used in these experiments, was able to bind to the receptor, we performed cell surface IL 1-receptor crosslinking using the murine thymoma line EL4 NOB.1.[18] Analysis of receptor/ligand complexes (Fig. 4) revealed a major band at approximately 95 kDa. Binding was fully displaceable by excess cold IL 1β but not by cold TNF α.

Production of IL 1β Binding Protein by Activated Mononuclear Cells

To identify the cell source of the soluble IL 1β binding protein, supernatants from resting and activated cells were analyzed by ^{125}I IL 1β covalent crosslinking and SDS-PAGE. Results obtained with normal human PBMNC showed that resting PBMNC did not produce the binding protein (Fig. 5) and stimulation with concentrations of LPS optimal for monocyte activation (100 ng/mL) also produced little binding activity. However, PHA concentrations optimal for PBMNC proliferation (1 μg/mL) induced production of the binding protein within 24 hr of culture and levels were maintained for up to 7 days. Stimulation of PBMNC with IL 1α at concentrations that induced both IL 1β and IL 6 production (10 ng/mL) failed to induce any binding protein activity. Culture of PB neutrophils in either

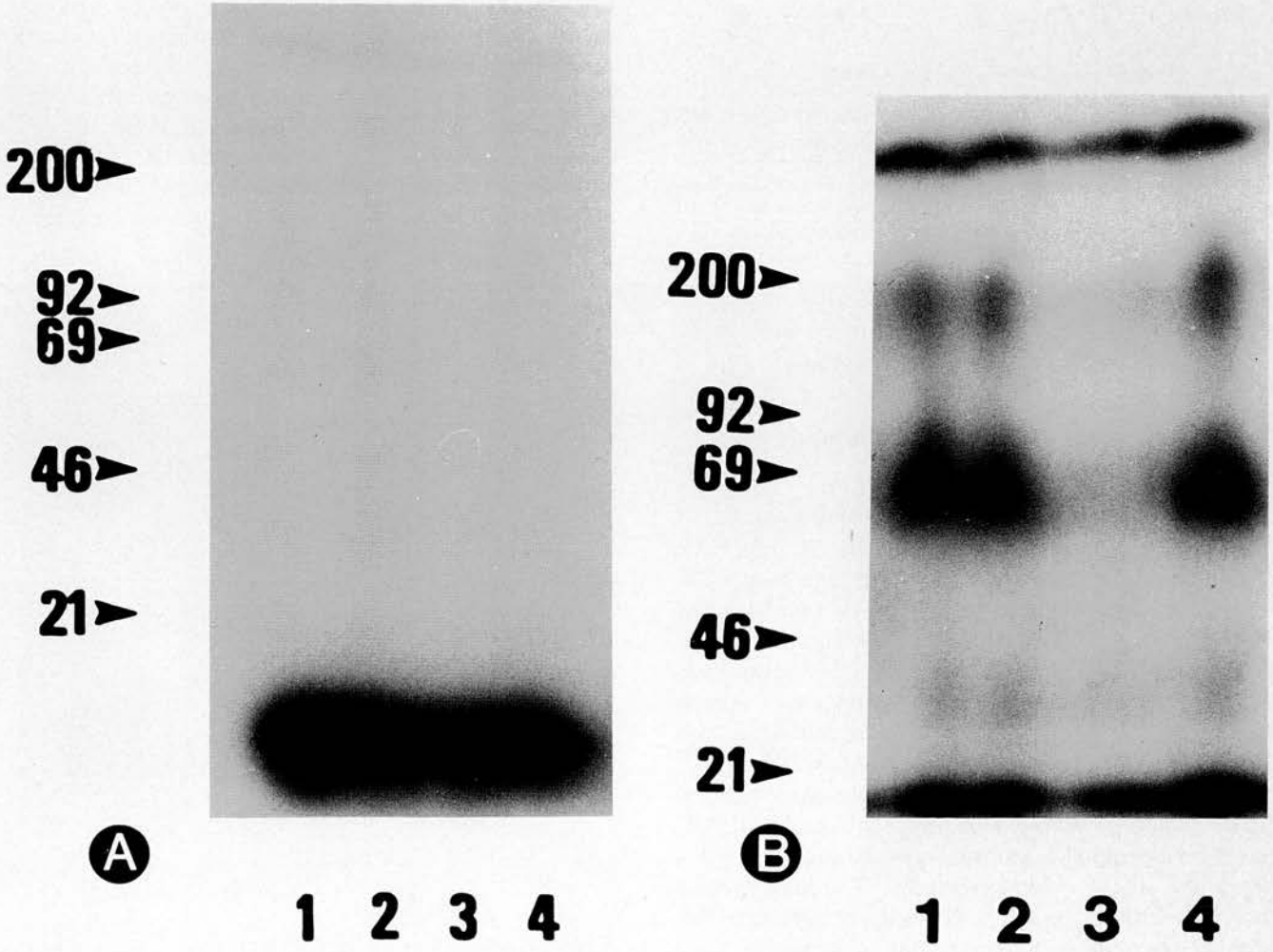

Figure 2. Analysis of IL 1 binding proteins in synovial fluid by soluble covalent cross-linking and SDS-PAGE.

Synovial fluids were incubated at 4°C overnight with (A) ^{125}I IL 1α or (B) ^{125}I IL 1β. Complexes were covalently cross-linked with DSS and subjected to SDS-PAGE. ^{125}I IL 1/binding protein complexes were identified by autoradiography. In each case Lane 1 is with no competing agent, Lane 2 with excess cold IL 1α, Lane 3 with excess cold IL 1β and Lane 4 with excess cold TNF α. Protein molecular weight markers are in kDa.

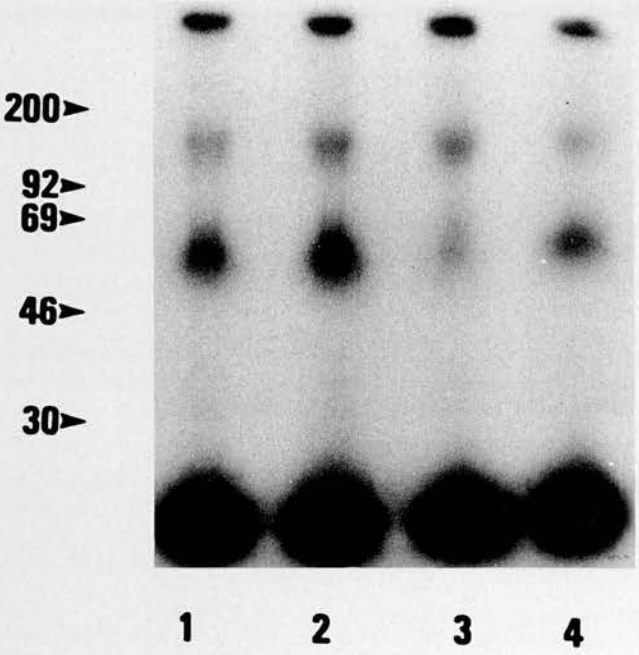

Figure 3. Analysis of IL 1 binding proteins in serum.

Serum from normal individuals was incubated with ^{125}I IL 1β at 4°C overnight. Subsequently complexes were covalently cross-linked with DSS and subjected to SDS-PAGE. ^{125}I IL 1β/binding protein complexes were identified by autoradiography Lane 1 with no competing agent, Lane 2 with excess cold IL-1α, Lane 3 with excess cold IL-1β and lane 4 with excess cold TNF α. Protein markers are in kDa.

media or with serum-treated zymosan resulted in no production of binding protein (data not shown).

Specific Binding of ^{125}I IL 1 to Soluble IL 1 Binding Proteins

We first tested the ability of solubilized EL 4 NOB.1 cell membranes to bind ^{125}I IL 1α (Fig. 6A). Maximal binding was achieved within 60 min at 22°C (data not shown). The binding of ^{125}I IL 1 alpha to the solubilized receptor was specific and saturable. Figure 6A shows ^{125}I IL 1α binding to a solubilized receptor as a function of ligand concentration. Saturation was seen at concentrations of 100 pM and above and Scatchard analysis (inset) indicated an apparent kD of 168 pM.

Soluble IL 1β binding protein was semi-purified from pooled synovial fluid by sequential DEAE-Sephacel ion exchange chromatography and wheat germ agglutinin affinity chromatography.[16] The semi-purified material retained the same specificity of binding as the original unpurified protein (data not shown). Time course studies of ^{125}I IL 1β binding to the semi-purified material revealed similar kinetics to the solubilized IL 1 receptor preparations. At 22°C maximal binding occurred within 60 min and saturation was achieved at concentrations above 200 pM ^{125}I IL 1β. Scatchard analysis (inset) revealed an apparent kD of 370 pM (Fig. 6B).

DISCUSSION

The present study describes the presence of a specific binding protein for IL 1β in normal human serum, inflammatory exudate and activated PBMNC culture supernatants. We have previously described the presence of this protein in normal human plasma.[17] The protein forms complexes with ^{125}I IL 1β, that elute at approximately 100 kDa on gel filtration and migrate at 60 kDa on SDS-PAGE. The binding of ^{125}I IL 1β to this protein is only competable by the addition of excess cold IL 1β and not by excess IL 1α or other cytokines, suggesting it is specific for IL 1β. To identify the cell population that produces the binding protein we cultured PBMNC for up to 7 days with media, IL 1α, LPS or phytohemagglutinin (PHA). Only stimulation with PHA resulted in synthesis of the binding protein. Production occurred within the first 24 hr and levels remained stable in the media for the 7 day culture period. Ligand-binding studies revealed that the soluble binding protein had a similar affinity for IL 1β as for the cell surface IL 1R and solubilized 80 kDa IL 1R.

Several IL 1 inhibitory molecules have been described,[8] although the precise mechanism of inhibi-

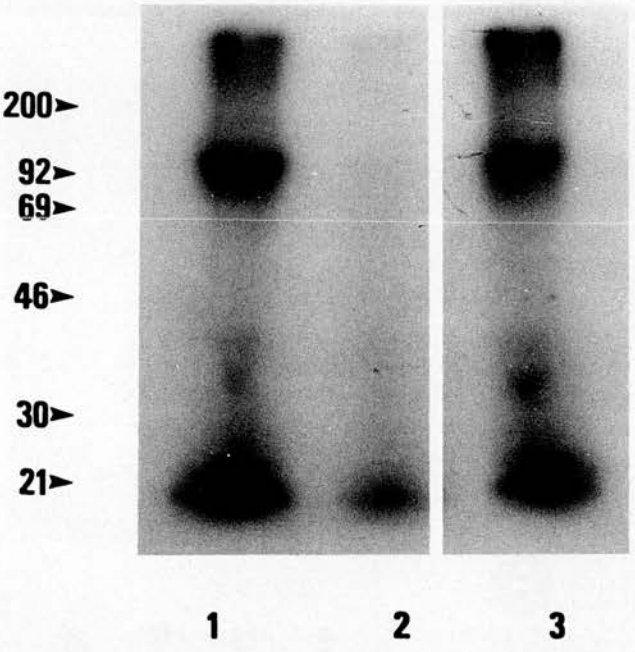

Figure 4. Affinity cross-linking of ^{125}I IL 1α to EL 4 NOB.1 cells.

EL 4 NOB.1 cells (1×10^7) were incubated with iodinated IL 1α. After washing, receptor-ligand complexes were crosslinked with DSS. Cells were lysed in 1% (wt/vol) SDS containing protease inhibitors and subjected to SDS-PAGE under reducing conditions. Lane 1 with no competing agent, Lane 2 with excess cold IL 1β, Lane 3 with excess cold TNF α. Protein markers are in kDa.

CYTOKINE, Vol. 2, No. 3 (May 1990: 190-198)

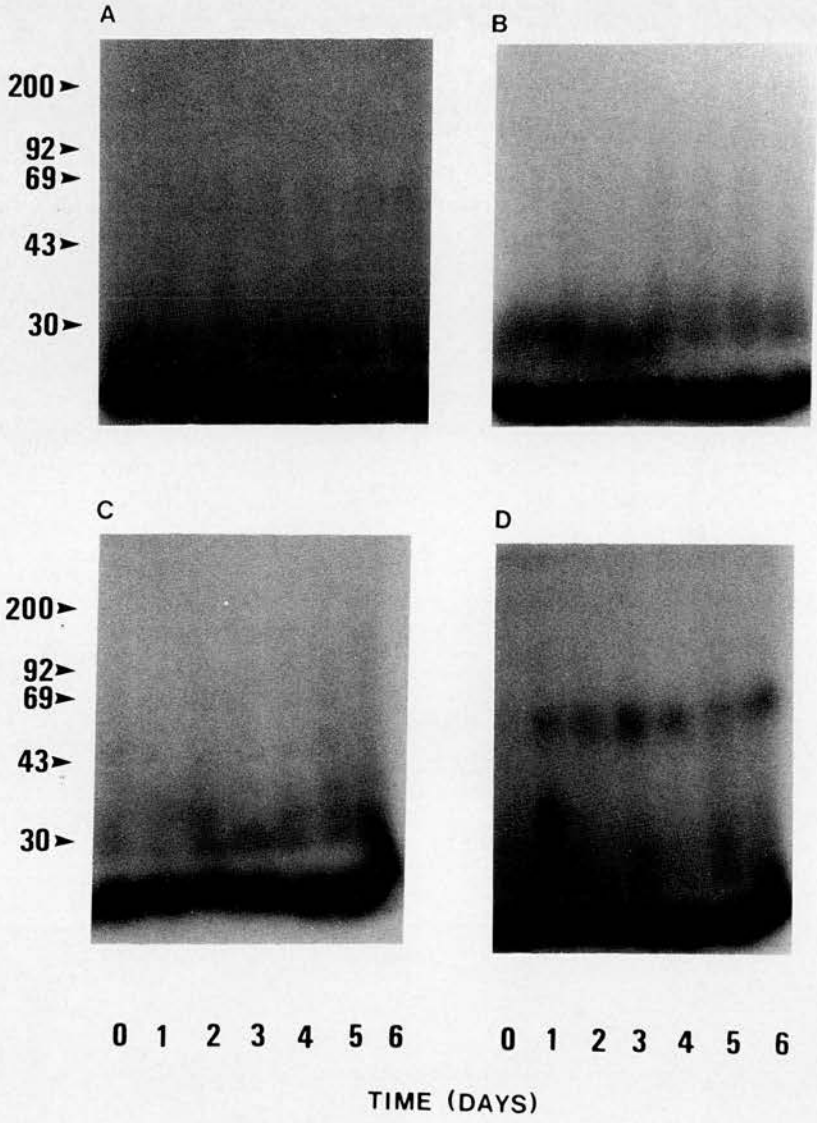

Figure 5. Production of ^{125}I IL 1β binding proteins by PBMNC.

PBMNC (2×10^6/mL) were cultured in serum-free RPMI 1640 for up to 7 days. Cells either remained unstimulated (a) or were stimulated with optimal concentrations of (b) LPS (100 ng/mL), (c) IL 1α (10 ng/mL) or (d) PHA (1 μg/mL) cell-free supernatants were incubated with ^{125}I IL 1β, cross-linked with DSS and analyzed by SDS-PAGE. Protein markers are in kDa.

tion in most cases is unknown. Prostaglandin E_2 inhibits monocyte IL 1 production posttranscriptionally,[14] by increasing intracellular levels of cAMP. Recently, an IL 1-receptor antagonist (IL 1rα) has been purified[9] and cloned.[10] It is produced by monocytes stimulated with IgG,[11] has 26% amino acid homology with IL 1β and blocks IL 1α and β action by binding to the 80 kDa IL 1 receptor. Unlike the binding protein described here the IL 1rα does not bind to ^{125}I IL 1.[9] An independent mechanism of IL 1 regulation may involve binding proteins, α_2 M, a major plasma proteinase inhibitor, is involved in the binding of cytokines including IL 6[19] and IL 1.[15] Binding to α_2 M is mediated through a thiol disulphide exchange reaction and, in each case, cytokine binding was demonstrated to be specific. However, it appears that α_2 M does not function as a biological inhibitor, as α_2 M/cytokine complexes retain biological activity.[15,19] In the case of IL 6, α_2 M was shown to protect the cytokine from the action of proteases such as trypsin and cathepsin G. α_2 M may, therefore, act as a carrier molecule and prevent the loss of cytokines from the circulation. The molecular weight differences between α_2 M and the IL 1β binding protein described here suggest that they are distinct. It is possible that the smaller IL 1β binding protein represents a fragment of α_2 M. However, unlike the binding protein described here, α_2 M binding to IL 1β occurs in the absence of covalent crosslinking agents and is retained on SDS-PAGE in native conditions.

It is now known that some cytokine receptors can exist in a soluble form. Initially it was demonstrated that the IL 2 receptor (IL 2R) α chain[21] could exist in a soluble form (sIL 2R). The mechanism of release appears to occur predominantly by proteolytic cleavage of the receptor molecule at the cell surface[20] and as sIL 2R retains ligand affinity,[36] it may function as an IL 2 inhibitor.[22,23] It is now known that IL 6, γ IFN,[24] TNF,[25] EGF[26] and the CSF 1[27] receptors can also exist in

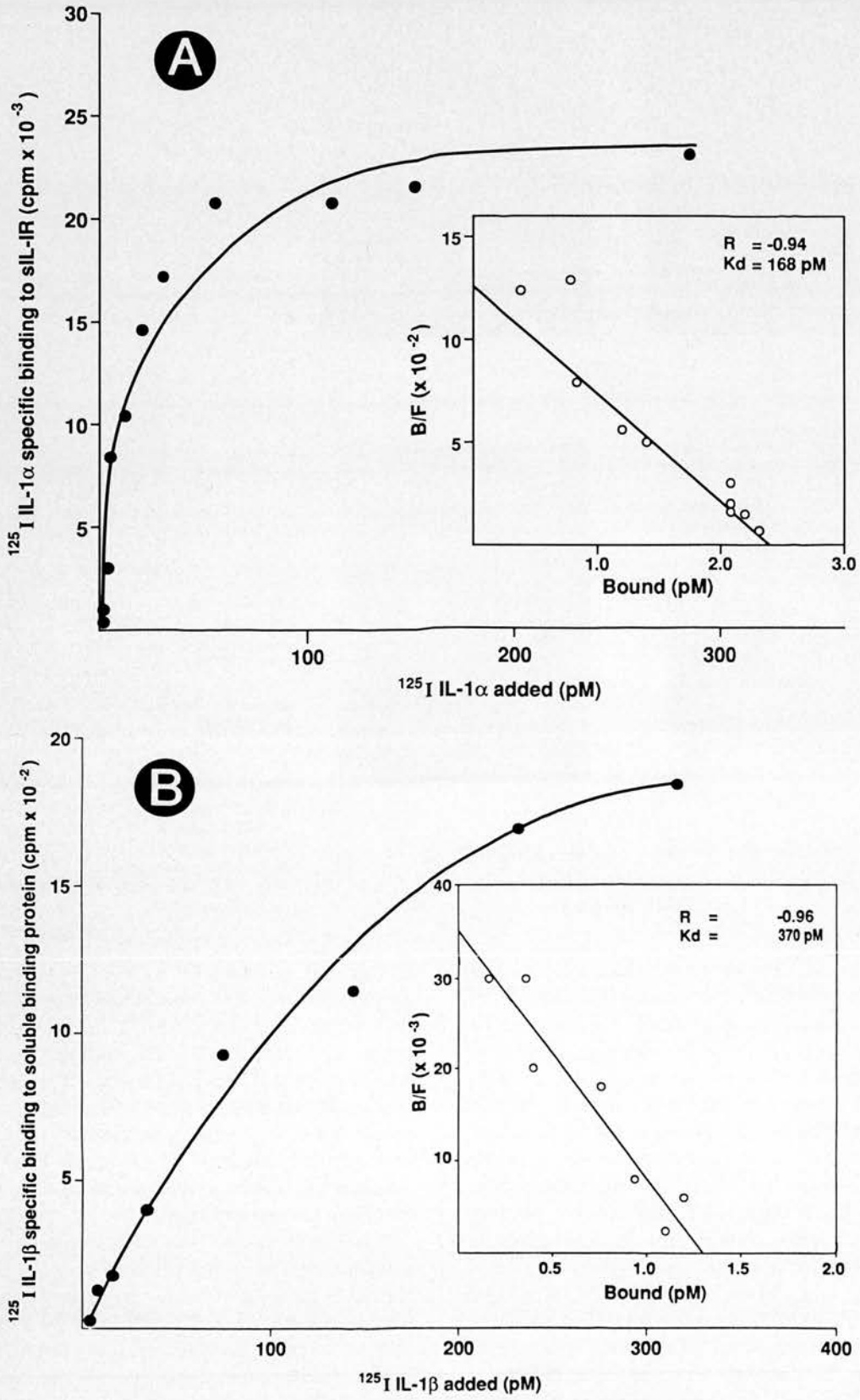

Figure 6. Specific binding of ^{125}I IL 1 to soluble IL 1 binding proteins.

EL 4 NOB.1 cell membranes solubilized with 8 mM CHAPS (A) or semi-purified IL 1β binding proteins from synovial fluid (B) were incubated with varying concentrations of ^{125}I IL 1 for 2 hr at 20°C and ligand-receptor complexes precipitated with polyethylene glycol. Binding shown represents specific binding. Scatchard analysis (insert) give Kd of approximately (a) 168 pm and (b) 370 pm. Results are representative of two experiments.

CYTOKINE, Vol. 2, No. 3 (May 1990: 190-198)

soluble form. The molecular weight, ligand affinity and cellular source of the IL 1β binding protein are consistent with the protein being a shed form of the 80 kDa IL 1R. Cloning of the human[6] and murine[5] IL 1R reveals that the extracellular domain consists of approximately 320 amino acids with seven potential N-linked glycosylation sites. The cross-linked ^{125}I IL 1β/binding protein complex migrates at approximately 60 kDa on SDS-PAGE suggesting that the binding protein may be approximately 43 kDa. Digestion of the complex with N-glycanase results in a band migrating at approximately 47 kDa (unpublished data) suggesting that the binding protein may have a molecular weight of 30 kDa with 13 kDa of N-linked carbohydrate. However, Dower et al.[28] have shown that recombinant murine sIL 1R, consisting of the extracellular region of the mature cell surface IL 1R, retains affinity for both IL 1β and IL 1α.

Recently, covalent labeling and ligand binding studies have revealed important differences between the IL 1R on T cell lines and B cell lines.[29] Covalent labeling of B cell lines with ^{125}I IL 1 results in the formation of a 65 to 70 kDa complex. Additionally, some B cell lines appear to bind IL 1α at a lower affinity than IL 1β. It has recently been shown that, in the mouse, these IL 1R proteins are encoded by distinct genes.[30] It is therefore possible that the IL 1β binding protein reported here represents a soluble form of a novel human IL 1R protein.

The cell source of the IL 1β binding protein was found to be PHA-activated PBMNC. Resting PBMNC did not synthesize the binding protein, while PHA-activated cells consistently produced binding protein within 24 hr of stimulation. Concentrations of LPS, optimal for monocyte activation, occasionally induced binding protein production. However, production only occurred after 72 hr stimulation, resulting in lower levels of binding protein production possibly reflecting autologous stimulation of lymphocytes by activated monocytes. Peripheral blood neutrophils were not found to produce the binding protein even after zymosan-stimulation and therefore, it is unlikely that the binding protein is related to the neutrophil-derived IL 1 inhibitor, which is described by Tiku et al.[31] We are currently screening human lymphocyte cell lines for binding protein activity.

Given that the IL 1β binding protein has a similar affinity for IL 1β as the cell surface 80 kDa IL 1R, the binding protein may function as a specific inhibitor for IL 1β. This may be important given the differential distribution of IL 1α and IL 1β peptides. Several studies have demonstrated that IL 1α remains predominantly cell-associated, while more IL 1β is translocated extracellularly.[32,33] To unequivocally test whether the IL 1β binding protein is an effective inhibitor of IL 1 activity will require homogenous protein for use in IL 1 bioassays and receptor binding assays. To achieve this, we have recently purified the IL 1β binding protein to homogeneity from pooled synovial fluid by sequential ion exchange chromatography, IL 1β ligand-affinity chromatography and reverse-phase high performance liquid chromatography (HPLC)[16] with a view to N-terminal sequencing and cloning.

MATERIALS AND METHODS

Reagents

Radioiodinated human recombinant (hr) IL 1α and hr IL 1β were obtained from Du Pont (NEN Products). IL 1α was radioiodinated using the chloramine-T procedure to a specific activity of 90.4 μCi per μg (3.35 MBq/μg). IL 1β was labeled with Bolton Hunter reagent to a specific activity of 158 μCi/μg (5.85 MBq/μg). Human recombinant IL 1 peptides were a gift from Biogen SA, human recombinant TNF α was a gift from Dr G. Adolf, Vienna, Austria. IL 1 and TNF α recombinant peptides were regularly assessed for bioactivity using the EL 4 NOB.1/CTLL-2 conversion assay and L929 cytotoxicity assay, respectively. The covalent cross-linking reagent disuccinimide suberate (DSS) was obtained from Pierce and Warriner Ltd.

Synovial fluid effusions were taken into ethylene diaminotetricetic (EDTA) tubes and blood into dry glass tubes for serum collection. To remove cells from the synovial fluid and sera, samples were centrifuged at 10,000 xg aliquoted and stored at −20°C until used. Synovial fluid aliquots were treated with hyaluronidase (bovine testes, type VIII; 100 U/mL, 45 min, 37°C; Sigma), followed again by centrifugation at 10,000 xg.

Gel Filtration Analysis

Synovial fluid (1 mL) was incubated with ^{125}I IL 1α (3 ng/mL), ^{125}I IL 1β (1 ng/mL) or ^{125}I IL 1β (1 ng/mL) plus cold IL 1β (500 ng/mL) at 20°C for 90 min. The fluid was then fractionated by gel filtration through a 1.5 × 90 cm Sephacryl S-200 column (Pharmacia) equilibrated with PBS, pH 7.2. One millilitre of sample was applied and eluted with a buffer flow rate of 1 mL/min. Fractions of 1 mL were collected and radioactivity assessed by γ-scintillation counting. The column was calibrated with marker proteins of known molecular weight. Data was expressed as a percentage of total counts applied to the column.

Covalent Crosslinking and SDS-PAGE Analysis

Synovial fluid, serum or culture supernatant (50 μl) was incubated overnight at 4°C with ^{125}I IL 1α or ^{125}I IL 1β at a final concentration of 10 ng/mL with or without 100-fold excess of cold IL 1α, IL 1β, or TNF α. Subsequently, DSS freshly prepared in DMSO, was added to a final concentration of 1 mg/mL and the sample incubated at 4°C for 45 min. Crosslinking reactions were terminated by the addition of 50 μl SDS-PAGE sample buffer (1% [wt/vol]) sodium dodecyl sulphate [SDS], 10% [vol/vol] glycerol, 62.5 mM Tris-HCl, pH 6.8). Samples, to be tested under reducing conditions, were treated with dithiothreitol (100 mM; DTT) and boiled for 10 min. Cross-linked complexes were identified in 10% poly-

acrylamide gels containing SDS[34] by autoradiography (Hyperfilm MP; Amersham). After 16 to 24 hr exposure the 17 kDa ^{125}I IL 1 band was removed and the film re-exposed for 24 hr to 4 weeks. Iodinated cytokines were assessed for self-association in all cross-linking experiments.

Isolation of PBMNC and Cell Culture

PBMNC were obtained from heparinized peripheral blood by density gradient centrifugation (pyrogen-tested lymphoprep; Nyegaard and Co). After washing, the cells were resuspended at 2.5 × 10^6/mL in serum-free RPMI 1640, containing penicillin (100 IU/mL), streptomycin (100 μg/mL) and L-glutamine (2 mM; Northumbria Biologicals [NBL]). The cells were then distributed in 200 μl aliquots in 96-well microtiter plates (Costar, NBL). PBMNC were maintained at 37°C in a humidified 5% CO_2/95% air atmosphere for up to 7 days with or without a previously determined optimal concentration of PHA (1 μg/mL; Sigma), LPS (100 ng/mL; E Coli 0127:B8, TCA extracted, Sigma) or hr IL 1α (10 ng/mL; Biogen). At the end of the culture, the medium was aspirated, centrifuged at 10,000 xg and stored at −20°C, until used in cross-linking experiments.

Cell Surface Affinity Crosslinking

EL 4 NOB.1 cells[18] were maintained in RPMI 1640 containing 5% (vol/vol) fetal calf serum (FCS). For binding assays, cells were harvested and washed 3 times in binding buffer (RPMI 1640 containing 1% [wt/vol] bovine serum albumin, 0.1% [wt/vol] sodium azide and 20 mM HEPES pH 7.2). Cells (1 × 10^7) were incubated with ^{125}I IL 1α (1 ng) in 150 μl of binding buffer alone or binding buffer containing 100-fold excess unlabeled IL 1 or TNF α for 2 hr at 4°C, with constant gentle agitation. The cells were subsequently washed twice in ice cold protein-free buffer and resuspended in 100 μl of the same buffer. DSS in DMSO was added to a final concentration of 1 mg/mL and incubated at 4°C for 45 min. The cells were washed twice in ice cold buffer and resuspended in 100 μl PBS, containing 1% (wt/vol) SDS, with the protease inhibitors phenyl-methylsulphonyl fluoride, pepstatin, o-phenanthroline and EDTA (all 2 mM; Sigma). After a 10 min incubation at 4°C, the samples were centrifuged at 10,000 xg and the supernatants stored at −20°C until SDS-PAGE analysis. An equal volume of SDS-PAGE sample buffer containing 100 mM DTT was added to an aliquot of the cell extract and the sample boiled for 10 min, and then subjected to electrophoresis in 10% SDS-PAGE gels, as described previously.

Soluble ^{125}I IL 1 Binding Assay

Soluble binding assays were performed on two sources of IL 1 binding proteins. 3-([3-cholomidopropyl] dimethyl ammonio)-1-propanesulfonate (CHAPS) solubilized EL 4 NOB.1 membranes were prepared, as described previously.[35] Semipurified IL 1β binding protein from synovial fluid was prepared by sequential ion exchange chromatography on DEAE Sephacel (Pharmacia LKB) and wheatgerm agglutinin affinity chromatography (Pharmacia LKB).[16,17] For soluble binding assays, IL 1 binding protein preparations were incubated with various concentrations of ^{125}I IL 1 in a final volume of 0.2 mL in Hank's balanced salt solution, supplemented with 20 mM HEPES, 1 mg/mL bovine serum albumin (Sigma; Cat. No. A7906) and 0.02% (wt/vol) sodium azide, pH 7.4. For assays with CHAPS solubilized EL-4 NOB.1 membranes, the final CHAPS concentration was adjusted to 0.8 mM. Nonspecific binding was assessed by inclusion of 100-fold excess cold IL 1. After incubation at 20°C for 2 hr, tubes were placed on ice and bovine γ globulin in PBS, added to a final concentration of 0.04% (vol/vol), and followed by 1.33 mL of 12% (wt/vol) PEG-8000 in PBS (Sigma). After vortexing, tubes were placed on ice for 10 min. Receptor/protein-bound ^{125}I IL 1 was separated from free ^{125}I IL 1 by first diluting the PEG-precipitated complexes with 3 mL cold 10% (wt/vol) PEG and filtering the entire volume under vacuum onto GF/C glass fibre filters (Whatman), mounted in a Millipore 1225 sampling manifold (Millipore). Filters were washed twice with 3 mL of 10% PEG solution, allowed to air dry and counted in a gamma counter. All experiments were performed in duplicate and results were expressed as specific binding activity after subtraction of non-specific binding.

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PLASMA LEVELS OF INTERLEUKIN 1 ALPHA IN RHEUMATOID ARTHRITIS

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SUMMARY

Interleukin 1 (IL1) beta has been implicated as an inflammatory mediator in rheumatoid arthritis (RA) but little is known about the related cytokine, IL1 alpha, in this disease. IL1 alpha has biological properties similar to IL1 beta but, unlike IL1 beta, remains mostly cell-associated. In this study plasma IL1 alpha was measured by radioimmunoassay in patients with RA and in healthy controls. Plasma levels were compared with conventional measures of disease activity. The mean levels in the two groups were not significantly different and, within the patient group (n=53), the only significant cross-sectional correlation was between plasma IL1 alpha and ESR. In longitudinal studies, some individual patients had plasma IL1 alpha levels that correlated with different measures of disease activity. We conclude that while IL1 alpha may be involved in the immunopathogenesis of RA, its measurement in plasma seems to offer little of clinical value.

Keywords Interleukin 1 alpha
Plasma
Rheumatoid arthritis
Prospective longitudinal study

INTRODUCTION

The two forms of IL1, alpha and beta, have similar receptor affinity (1) and biological activities in inflammatory and immune reactions (2). They are produced by different genes with inducible expression in macrophages and other cell types. The initial translation product, a 31KDa propeptide, is processed to produce biologically active 17KDa forms (3,4). The 31KDa IL1 alpha propeptide is also biologically active.

The activities of IL1 suggest a pathogenic role in rheumatoid arthritis (RA) (5). In synovial cells, IL1 augments the release of vasoactive agents and mediators of tissue damage (6,2), it also stimulates bone (8) and cartilage resorption (7), the acute phase response (9) and fever (10). IL1 may also contribute to chronic inflammation by inducing lymphocyte growth factors and their receptors (e.g. IL2, IL6, IL2 receptor) (11,12,13).

Evidence for IL1 involvement in RA includes: high levels in synovial exudates (14); spontaneous production in vitro by synovial cells (15) and detection of IL1 mRNA in synovial tissue sections (16); in animals, intra-articular injections of IL1 produced synovial fluid leucocytosis and cartilage proteoglycan breakdown (17). Recently, a simple extraction procedure enabled detection of IL1 beta in plasma (18), and showed raised levels in RA that correlated with conventional laboratory and clinical measures of disease activity (19). Interleukin 1 alpha has been less well studied and we have now measured plasma IL1 alpha in patients with RA and in healthy volunteers. To our knowledge this is the first report of immunoreactive IL1 alpha in the plasma of RA patients.

METHODS

Patients with definite or classical RA (20) attending an out-patient clinic or admitted to hospital with active disease were graded for disease activity by Ritchie joint index (21), duration of early morning stiffness, and visual analogue pain score. At the same time blood was taken for haemoglobin

concentration, white cell count, platelet count, erythrocyte sedimentation rate (ESR) and rheumatoid factor titer as part of normal management. Blood samples for plasma preparation were collected into EDTA with aprotinin, separated and stored as previously described (19). All patients received non-steroidal anti-inflammatory drugs (NSAID) and some received anti-rheumatic drugs including intra-articular steroids.

IL1 alpha measurement

Samples were extracted according to the method of Cannon et al (18). Treated plasma was tested in a specific radioimmunoassay (RIA) for IL1 alpha. The assay is sensitive to 78pg/ml IL1 alpha, has parallel dilution characteristics and less than 10% interassay variability. Recovery (detection) of recombinant IL1 alpha added to RA plasma was 87% after extraction (n=8, mean 87.1% SEM = 6.7%). The difference in recovery of IL1 alpha added to RA plasma compared with normal plasma was not significant. There is no cross reactivity with IL1 beta. The RIA was developed by Amersham International, (Bucks, UK). Samples or standards were incubated for 4 hours with rabbit anti-human IL1 alpha antiserum. ^{125}I-IL1-alpha tracer (human recombinant) was then added and incubated overnight at 4° C. Donkey, anti-rabbit serum coated on magnetizable polymer particles (Amerlex-M) was added for 10 minutes at room temperature. After centrifugation, supernatant was removed and the pellet counted by gamma scintillation. The standard curve was prepared with human recombinant IL1 alpha.

Statistical Methods

Mean values were compared by student's t-test and correlations assessed with the Spearman rank correlation coefficient.

RESULTS

IL1 alpha levels in plasma

In testing the extraction procedure, 17 normal plasma samples showed a statistically non-significant increase in the level of IL1 alpha (unextracted mean IL1-alpha = 222pg/ml, extracted mean IL1-alpha = 249pg/ml, p=0.08). However, in the same experiment, 44 samples from RA patients showed a small but significant increase in plasma IL1-alpha after extraction (unextracted mean IL1-alpha = 192pg/ml, extracted mean IL1-alpha = 238.5pg/ml, R=0.535, p=0.0003).

Between the patient and control groups there was no significant difference in the plasma IL1 alpha levels. (Table I) Mean ages of the two groups were 40 years (20-63) and 38 years (26-54) respectively.

Cross-sectional studies

Plasma IL-1 alpha levels in 53 RA patients were compared with conventional measures of disease activity and also with IL1 beta levels. The only significant, but weak, correlation found was between plasma IL-1 alpha and ESR (R=+0.29, $p<0.05$). No obvious relation could be seen between IL1 alpha and drug treatment.

Longitudinal studies

Serial measurements of IL1 alpha in plasma taken from 3 hospitalised patients over a period of several weeks were compared with conventional measures of disease activity (Fig. 1). Patient A showed a gradual reduction in disease activity over the first 15 days but then had a secondary flare in disease activity. Plasma IL1-alpha in this patient correlated positively with Ritchie joint score (R= +0.678, $p<0.005$), analogue pain score (R= +0.59, $p<0.02$), duration of morning stiffness (R= +0.538, $p<0.05$) and negatively with hemoglobin concentration (R= -0.49, $p<0.05$). Other correlations were not significant. Patient B showed significant correlations between plasma IL1 alpha and duration of morning stiffness (R= +0.587, $p<0.02$) and with platelet count (R= +0.719,

$p<0.005$). Patient C showed similar trends though none reached statistical significance.

DISCUSSION

IL1 alpha was present at high levels relative to IL1 beta in both control and patient populations (19). There was no significant difference in mean plasma IL1 alpha levels between the two. There was no cross-sectional correlation of plasma IL1 alpha levels with any of the measures of disease activity except a weak relation to ESR. However, in two of the three patients tested, serial measurement of immunoreactive IL1 alpha in plasma correlated with some clinical and laboratory assessments of disease activity.

These results differ from those found for IL1 beta in plasma where extracted IL1 beta levels were significantly higher in the patient group compared with controls (100pg/ml vs 50pg/ml) and correlated with disease activity in cross-sectional population analysis (19).

Monocytes stimulated in vitro produce both IL1 alpha and IL1 beta. The latter is rapidly released (50% at 24 hours) with measurable extracellular levels after 2 hours. In contrast, IL1 alpha remains predominantly cell-associated (95% at 24 hours) and is not detected in supernatants until 12 hours after initiation of culture (22). It has therefore been proposed that IL1 alpha is a cell-associated form (23) and may be active on the cell surface. In contrast, IL1 beta appears to be the predominant secreted form mediating biological activity at a distance.

Other work suggests that different cell types vary in the ratio of IL1 forms that they produce and may also have different translocation patterns (24). Keratinocytes, for example, are reported to produce mainly IL1 alpha and it is secreted in culture (24).

Production of IL1 alpha by different cell types could explain the similar blood levels in patients and control groups. A large cell population such as the skin keratinocytes secreting predominantly IL1 alpha may be stimulated to a

comparable level in both populations (e.g. by exposure to UV). This would lead to detectable blood levels even in healthy individuals and increased production from cells at a site of inflammation might appear in the plasma as a relatively small increment on a high background.

IL1 alpha has been demonstrated in RA synovial fluid (12,25). Synovial cells in RA and blood cells in Still's disease expressing IL1 alpha mRNA have been detected by in situ hybridization (unpublished observations). Within individuals, activated synovial cells producing IL1 alpha may account for changing plasma levels with clinical disease activity. Our results give some support for a role of IL1 alpha in RA but suggest that its measurement in plasma is of limited clinical value.

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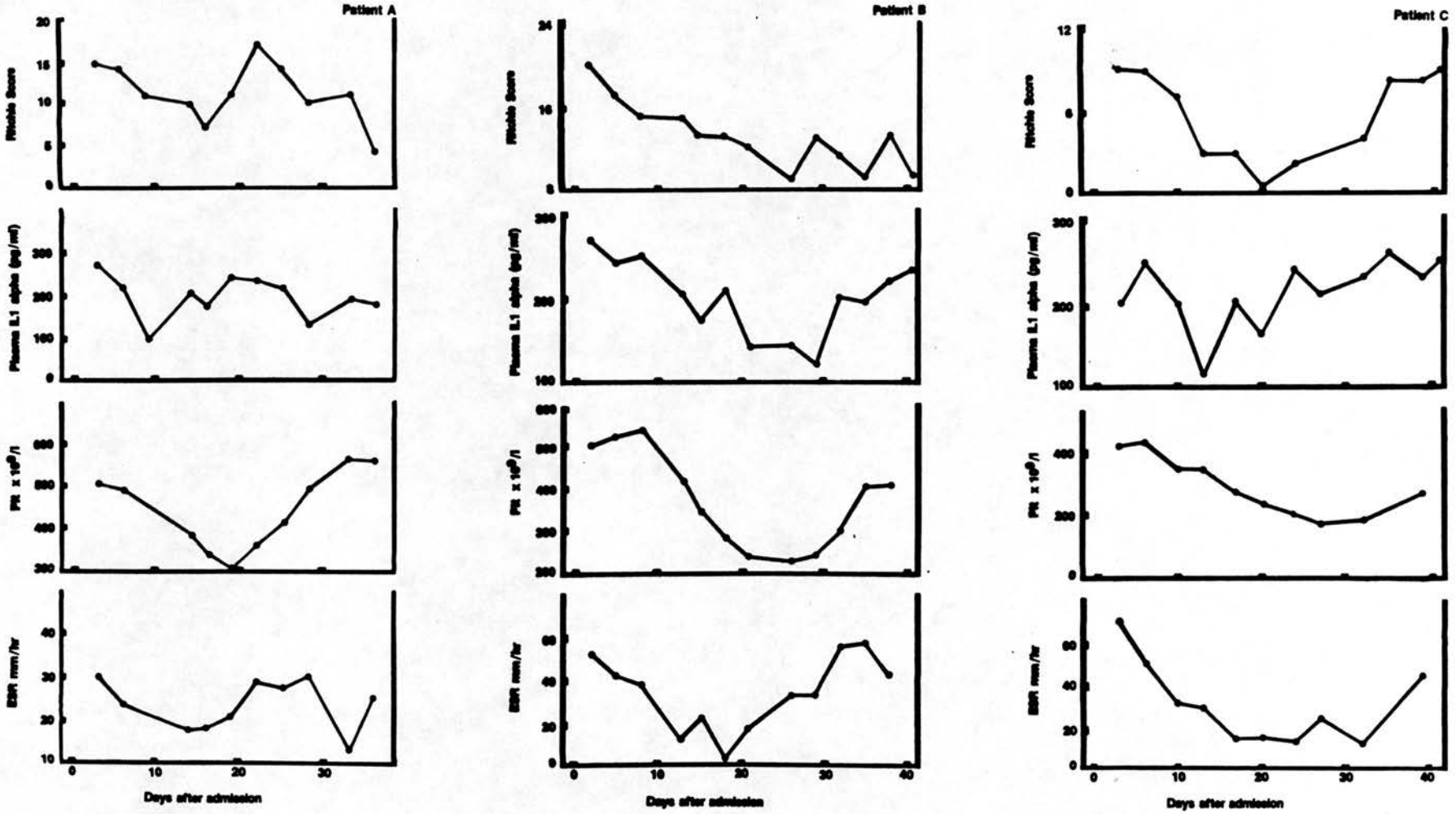
Table I **Plasma IL1 alpha levels in extracted plasma**

	Plasma IL1 alpha (pg/ml)	
	Control	RA
n	33	53
Range	110-620	110-500
Median	220.0	230.0
Mean	239.5	245.5
SEM	14.7	10.5
Controls vs RA	t=0.338	p=0.736

Figure 1

Serial measurements of plasma IL1 alpha in three RA patients during hospital admission with concurrent clinical and laboratory assessments of disease activity.


Patient A
Patient B
Patient C
Rankin Score
Plasma IL1 alpha (pg/ml)
Plt x 10⁹/l
ESR mm/hr
Days after admission